PLASMA 5-S-CYSTEINYLDOPA IN PHYSIOLOGICAL AND PATHOLOGICAL PIGMENTARY STATES

by

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DECLARATION OF ORIGINALITY

I declare that the work presented herein and the composition of this thesis are my own.

Judith E Nimmo

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I would like to dedicate this thesis to my family, and in particular, to my husband Graham for his encouragement and greatly appreciated moral support.

ABSTRACT

This thesis describes the development and subsequent use of a high-performance liquid chromatographic technique for the measurement of the melanin precursor 5-S-cysteinyldopa (5-S-CD) in plasma, using its diastereomer, 5-S-D-cysteinyl-L-dopa as internal standard. The assay developed had a between-batch coefficient of variation (CV) of less than 4 percent, and was sufficiently sensitive to detect picogram quantities of 5-S-CD.

The method was used to assess the value of plasma 5-S-CD as a marker for the melanocytic tumour, malignant melanoma.

Study of healthy volunteers established an upper limit of normal for plasma 5-S-CD of 20nmol per litre, and also demonstrated the changes in plasma 5-S-CD which occurred in individuals throughout the year in Edinburgh. Skin pigmentation and hair colour did not influence plasma 5-S-CD levels, but exposure to ultraviolet radiation (UVR) in the form of photochemotherapy (PUVA) or short wave UVR therapy (UVB) was shown to increase plasma 5-S-CD levels dramatically in psoriatic patients.

Patients with a variety of tumours were studied. Plasma 5-S-CD levels were not raised in patients with primary melanoma or in patients with non-melanoma tumours, such as breast, lung, and stomach cancer. Elevated levels were found in 60 percent of patients with secondary melanoma, in some cases before the secondary tumour was detectable by clinical means.

Impaired liver function did not, in general, affect plasma 5-S-CD levels, but raised levels were seen in a high proportion of patients with impaired renal function. Raised levels were also found in patients with Parkinson's disease who were undergoing treatment with dopa and carbidopa.

The finding that patients with tyrosinase-negative oculocutaneous albinism had normal levels of plasma 5-S-CD led to

some consideration of possible non-tyrosinase mechanisms for the generation of this amino acid.

In conclusion, the measurement of plasma 5-S-CD was shown to be of use in the early detection of metastatic disease in a proportion of patients with malignant melanoma, but the value of this test was found to be limited by a number of factors, the most significant being the number of patients in whom the presence of secondary melanoma was not reflected by a raised plasma 5-S-CD concentration.

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INTRODUCTION

General Introduction

The main purpose of the work presented in this thesis was to study the amino acid 5-S-cysteinyldopa (5-S-CD) in plasma, and to examine its use as a tumour marker for malignant melanoma. 5-S-CD is an intermediate in the synthesis of melanin, and this introduction will, therefore, review melanin synthesis, before turning to malignant melanoma and summarising progress in the search for a specific and sensitive marker for this tumour.

Melanin Pigmentation and Skin Colour

Normal human skin colour depends upon the presence of four pigments; haemoglobin, oxyhaemoglobin, melanin and carotenoids. The pink appearance of the untanned Caucasoid is due to the pigment oxyhaemoglobin in the blood within the capillary plexus of the superficial dermis. Carotene, a yellow pigment, is found in the subcutaneous fat and epidermis. Skin pigmentation is the result of the balance between these factors and melanin, the major determinant of skin colour. Variation in the melanin pigmentation of the skin accounts for the wide spectrum of human skin colour (Hunter, 1977).

The word melanin is derived from the Greek μελας (melas), meaning black. In fact this group of pigments includes the black or brown nitrogenous eumelanins, the yellow or reddish brown sulphur-containing phaeomelanins, and other pigments whose physical and chemical properties are intermediate between those of the eumelanins and the phaeomelanins (Prota, 1980).

Melanins are found in virtually all groups of living organisms, including higher plants, fungi and bacteria. In the human, melanin is produced solely by cells known as melanocytes. These cells are present in the skin, the uveal tract of the eye, and the leptomeninges, having migrated to these positions from the neural crest early in foetal life (Rawles, 1948). In normal skin melanocytes are dendritic cells situated just above the basal lamina, and their dendrites project between the surrounding keratinocytes, situated in the lower epidermis (Fig. 1.1). Each

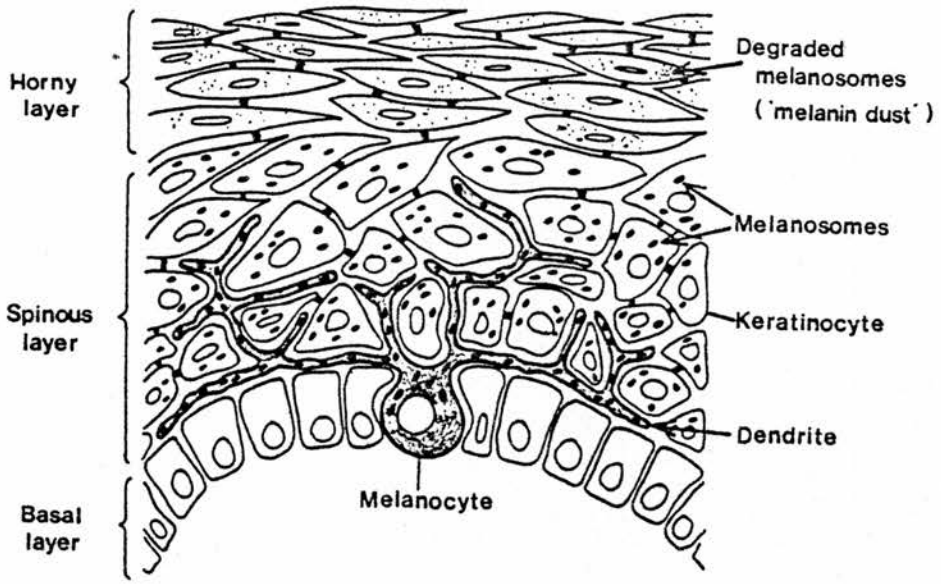

FIGURE 1.1 Diagramatic representation of the layers of the epidermis

melanocyte and its associated group of approximately 36 keratinocytes is known as an epidermal melanin unit (Fitzpatrick and Breathnach, 1963).

Within the melanocytes melanin is produced in specialised cytoplasmic organelles known as melanosomes; this was first demonstrated by Seiji et al. (1961) who were able to separate the subcellular components of the melanocyte by sucrose gradient ultracentrifugation. The enzyme tyrosinase is produced by the ribosomes of the melanocyte, as shown by the studies of Wellings and Siegel (1963). It is then transported through the endoplasmic reticulum (ER) to the Golgi system. From this area, or from the smooth ER, tyrosinase-containing vesicles bud off to form the ultrastructural precursor of the melanosome (Seiji and Iwashita, 1965; Hunter et al., 1970). Drochmans (1967) studied high resolution pictures of melanosomes and deduced that the first stage in formation of the melanosomes was the deposition of a protein matrix, composed of coiled filaments. Cross-linking of these filaments was thought to be responsible for the transverse striations visible in the micrographs. After formation of the protein matrix (possibly tyrosinase units or tyrosinase linked with a structural protein), melanin deposition gradually occurs, and the pigment accumulates on the inner membranes obscuring the characteristic periodicity (100Å) of the structure. Finally the organelle becomes a uniformly dense particle without discernible internal structure. The fully melanised melanosome appears under the electron microscope as a cigar-shaped, electron-dense organelle about 0·4 - 1·2 μm along its major axis, without obvious periodicity

(Hunter, 1977).

In the epidermis the melanosomes are passed from the melanocytes to their neighbouring keratinocytes by means of the melanocyte's dendritic processes (Cruickshank and Harcourt, 1964). The mechanism by which this occurs is not fully understood; originally it was thought that the melanosomes were injected into the keratinocytes, but it is possible that the keratinocytes phagocytose the tips of the dendrites. The melanosomes within the keratinocytes are seen to occur either as individual membrane-bound particles, or as aggregates of two or more particles within membrane-limited vesicles containing lysosomal enzymes (Szabo et al., 1969). Aggregated melanosomes within keratinocytes undergo gradual degradation into smaller electron-dense particles. The aggregation and degradation of melanosomes within keratinocytes is a size-dependent phenomenon since large single melanosomes do not appear to be broken down in this way. Wolff and Konrad (1972, 1973) have demonstrated this size-dependent complexing of melanosomes both experimentally (using latex beads in guinea-pig skin) and in human pigmentary disorders. Particles of 0·1 μm tended to be complexed whilst those with a diameter of 0·8 μm were not. Zaynoun and coworkers (1977) confirmed the size-dependence of melanosome aggregation in their studies of pigmentation induced by topical psoralens and coal tar.

Human skin contains around 2000 melanocytes per mm^2 in the face and genital regions, whereas the rest of the body has a density of around 1000 per mm^2 (Szabo et al., 1969). There is no significant

difference in the number of melanocytes per area of skin from one racial group to another (Billingham, 1949). Shades of brown-black pigmentation of skin, therefore, depend on factors other than simply the number of melanin-producing cells in the skin. There are fewer melanosomes in the melanocytes and keratinocytes of Caucasoids and Mongoloids than in those of Negroids. Of those present in the melanocyte most are in stages I, II and III of development (Fig.1.2), and are not fully melanised. Those in the keratinocytes are fully melanised but tend to be small and grouped in membrane-limited organelles to form 'melanosome complexes'. In the skin of Negroids and Australian aborigines there are more melanosomes in the melanocytes and keratinocytes than in Caucasoids, and a high proportion of melanosomes are in the final stage of development. Most of the melanosomes in the keratinocytes appear disposed individually rather than in complexes (Szabo et al., 1969).

Melanin pigmentation of human skin can be divided into constitutive and facultative skin colour (Quevedo et al., 1974). Constitutive skin colour is genetically determined, and is generally taken to be the degree of pigmentation found in those parts of the body habitually shielded from light, although true constitutive skin colour may only be seen in the newborn infant. Facultative skin colour is the increase in melanin pigmentation brought about by external influences, mainly exposure to ultraviolet radiation (UVR). Skin hyperpigmentation induced by endocrine changes such as those

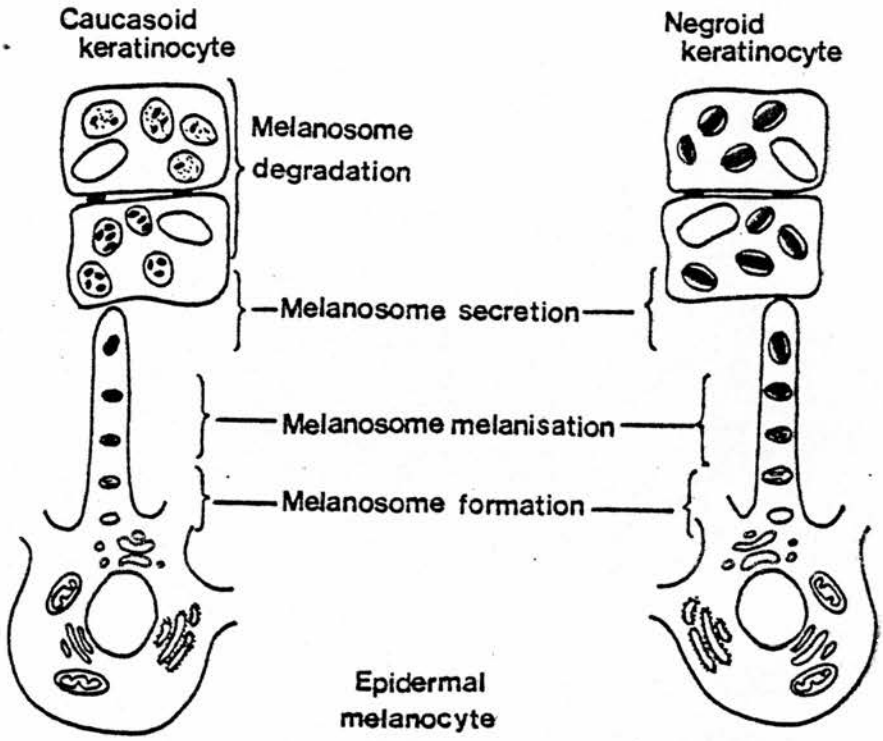

FIGURE 1.2 The epidermal melanin unit in caucasoid and negroid skin

occurring in pregnancy and Addison's disease is another type of facultative colour change. Stimuli causing facultative colour changes may interact as, for example, during alterations in endocrine balance (such as those seen in pregnancy), which may increase the response of human skin to UVR. The changes in pigmentation provoked by UVR can be divided into immediate tanning, occurring within minutes of exposure to UVR and disappearing rapidly, and delayed tanning, developing 48-72 hours after exposure. Immediate tanning is thought to involve a number of factors: a rapid transfer of melanosomes from the centre of the melanocytes to the dendrites, and from the dendrites of the melanocytes to the keratinocytes, by some system involving microfilaments and microtubules, rapid redistribution of melanosomes within keratinocytes, and temporary oxidation of melanosomes to a darker form (Quevedo et al., 1974). Delayed tanning is more complicated, the changes occurring being more numerous. These are thought to include:-

1) an increase in the number of functional melanocytes due to proliferation of melanocytes and possibly activation of dormant melanocytes (Rosdahl, 1978),

2) hypertrophy of melanocytes with increased arborization of dendrites,

3) an increase in melanosome synthesis (Zaynoun et al., 1977),

4) an increase in the rate of melanogenesis within the melanosomes,

5) an increase in transfer of melanosomes from melanocytes to keratinocytes,

6) activation of tyrosinase by a direct effect of UVR on the tyrosinase - inhibiting sulphydryl compounds in the epidermis, such as glutathione (Daniels and Johnson, 1974; Jimbow et al., 1974; Quevedo et al., 1974).

While the most effective stimulus to melanin production is exposure to short wave UVR (290-320 nm), long wave UVR and even visible light (320-700 nm) can also stimulate melanogenesis to a much lesser extent.

Hormones can also exert a marked influence on the melanin pigmentation of human skin, although a physiological role seems unlikely. Administration of α-melanocyte stimulating hormone (α-MSH) to human subjects, in pharmacological doses, has been shown to result in marked hyperpigmentation of the skin (Lerner and McGuire, 1961). Electron microscopic examination of the skin shows increased numbers of melanosomes and highly dendritic melanocytes, suggesting increased transport of melanosomes into keratinocytes, although the rapid dispersion of melanosomes readily demonstrated in fish, amphibians and reptiles has not been directly observed in mammals (Lerner, 1971). Although certain pituitary peptide hormones, such as MSH and adrenocorticotrophic hormone (ACTH), can influence human skin pigmentation when grossly elevated or given in pharmacological doses, it seems unlikely that these hormones have a significant influence on normal physiological melanin pigmentation (hypophysectomy does not result in lightening of negro skin; Gilkes, 1982).

Chemistry of Melanogenesis

The chemical processes leading to the formation of melanin are subjects in which there has been considerable interest over the past hundred years. In 1895 Bourquelot and Bertrand discovered tyrosinase in a black toadstool, Russula Nigricans. It soon became appreciated that the enzyme was widely distributed in the vegetable kingdom. In the early 1920s, Raper and his colleagues carried out extensive studies on the metabolic pathway involved in the conversion of tyrosine to melanin in plants. The pathway originally outlined by Raper (1928) has since been generally accepted, although some modifications have been suggested. Much of the melanin synthetic pathway in humans, however, remains to be elucidated. In the early stages, eumelanins and phaeomelanins are derived from the amino acid tyrosine, and have, in part a common synthetic pathway. The first two steps are catalysed by the enzyme tyrosinase and involve the conversion of tyrosine to dihydroxyphenylalanine (dopa), followed by the oxidation of dopa to the orthoquinone, dopaquinone (Fig.1.3). Orthoquinones are amongst the most reactive of organic compounds and are capable of undergoing a number of spontaneous reactions including oxidative polymerisation and condensation with $-NH_2$ or -SH groups of other molecules. The reactivity of quinone intermediates represents a potential hazard to the cell in which they are formed, and the cytotoxicity of tyrosine metabolites has been demonstrated (Pawelek and Lerner, 1978). In fact, any phenol compound represents a potential hazard to the melanocyte due to its selective uptake by

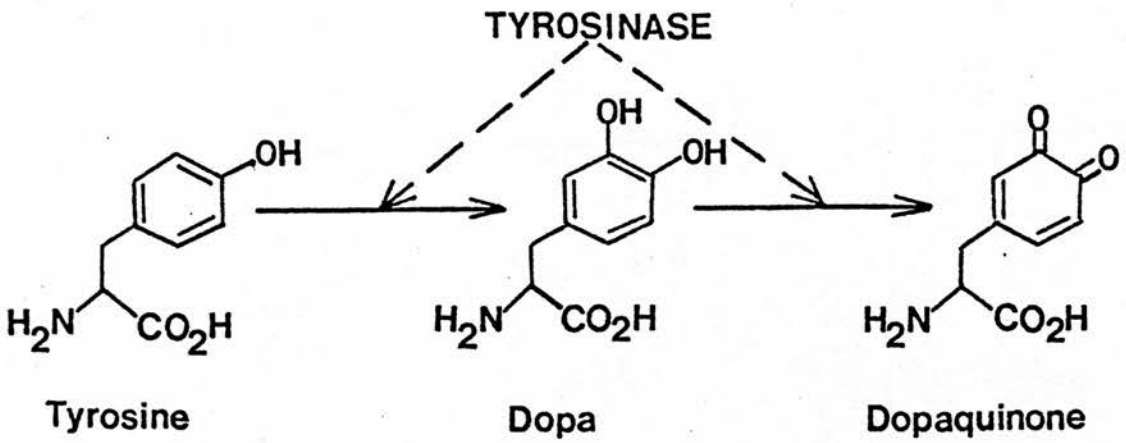

FIGURE 1.3 Conversion of tyrosine to dopaquinone by the action of tyrosinase

the cell and subsequent metabolism with generation of potentially damaging quinones. Pigment production, therefore, may be seen as a detoxification mechanism by which highly reactive quinones are converted to inert polymers by a series of spontaneous reactions.

The enzyme or enzymes involved in the conversion of tyrosine to dopaquinone have long been the subject of some controversy. Evidence has been produced for the involvement of two separate enzymes; peroxidase to catalyse the conversion of tyrosine to dopa and dopa oxidase to catalyse the oxidation of dopa to dopaquinone (Okun et al., 1973). More recent studies have, however, provided further evidence for the existence of a true tyrosinase in mammalian melanocytes, capable of catalysing both reactions (Hearing and Ekel, 1975; Smith and Swan, 1976). Copper has been reported to be an essential part of the tyrosinase molecule, and tyrosinase activity can be inhibited by substances which bind copper (Lerner et al., 1950). Tyrosinase is the only known enzyme involved in melanin synthesis. Once dopaquinone has been produced by the action of this enzyme, its reactivity causes the subsequent steps in the pathway to occur spontaneously. The further steps in eumelanin synthesis have been largely worked out by Raper and Mason (Raper, 1928; Mason, 1948) (Fig.1.4). Dopaquinone undergoes a spontaneous intramolecular cyclisation in which the amino group of the side chain rapidly adds to the quinone system to produce leucodopachrome. Leucodopachrome is then oxidised to dopachrome, which undergoes rearrangement, and decarboxylation, to produce 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-

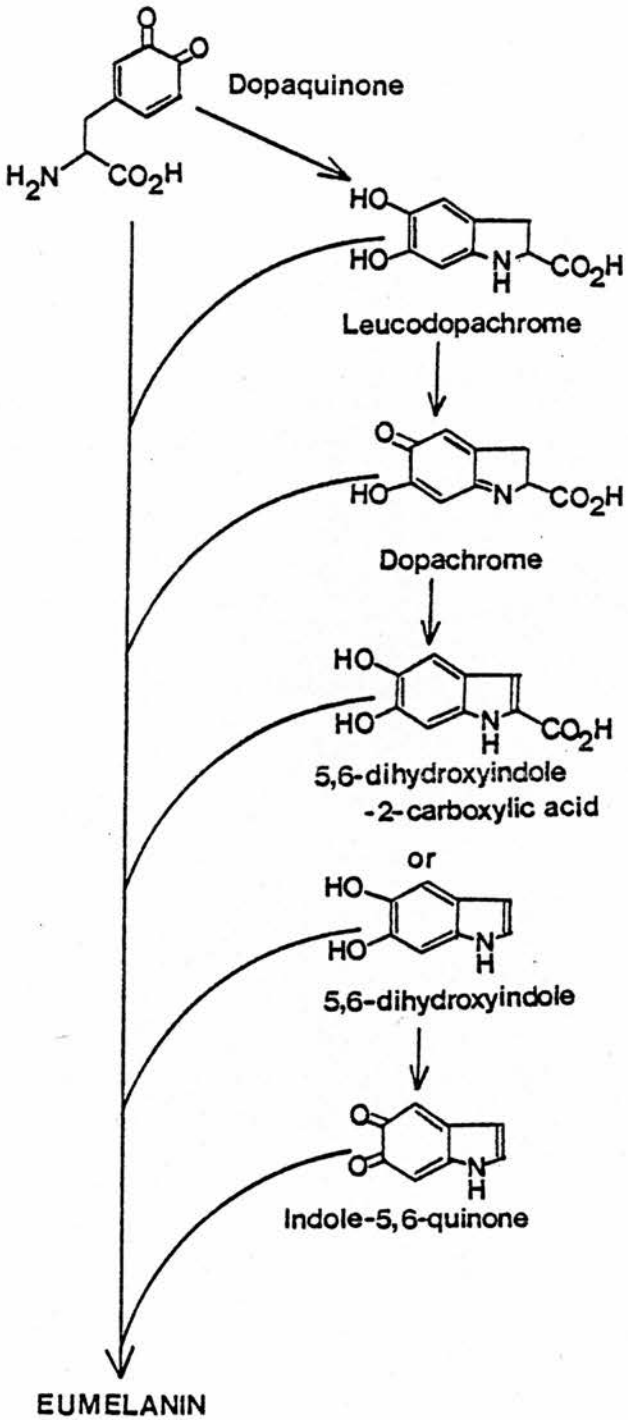

FIGURE 1.4 Eumelanin synthesis from dopaquinone

carboxylic acid. Oxidation of 5,6-dihydroxyindole leads to formation of the highly reactive intermediate 5,6-indolequinone, which, by repeated oxidative coupling gives rise to the eumelanin polymer. Study of the chemical properties of eumelanin, however, has shown that the process is not quite as simple as this, and it appears that not only the indolequinone, but also all of the other quinone and catechol intermediates can undergo polymerisation to give different melanin products.

The synthesis of phaeomelanin, rather than eumelanin, appears to depend solely on the presence of cysteine or glutathione residues in the cell (Prota et al., 1968; Ito and Prota, 1977). Cysteine contains the reactive sulphydryl group which is able to combine with the dopaquinone molecule at three possible sites on the ring system to produce four different cysteinyldopa adducts. (Fig.1.5). Three of these are produced by the addition of the thiol group of a single cysteine residue to the 2, 5 or 6 position of the dopaquinone ring system. The fourth cysteinyldopa arises by the addition of two cysteine molecules to the dopaquinone ring in the 2 and 5 positions. The major product of the addition of cysteine to dopaquinone is 5-S-CD, which makes up approximately 75 percent of the cysteinyldopas formed by this reaction (Prota et al., 1968; Ito and Prota, 1977). This molecule is consequently the main building stone for the phaeomelanins, and for the trichochromes, a group of pigments found in red or yellow hair or feathers. 5-S-CD can also be produced by the reaction of dopaquinone with glutathione followed by enzymatic hydrolysis of the resulting

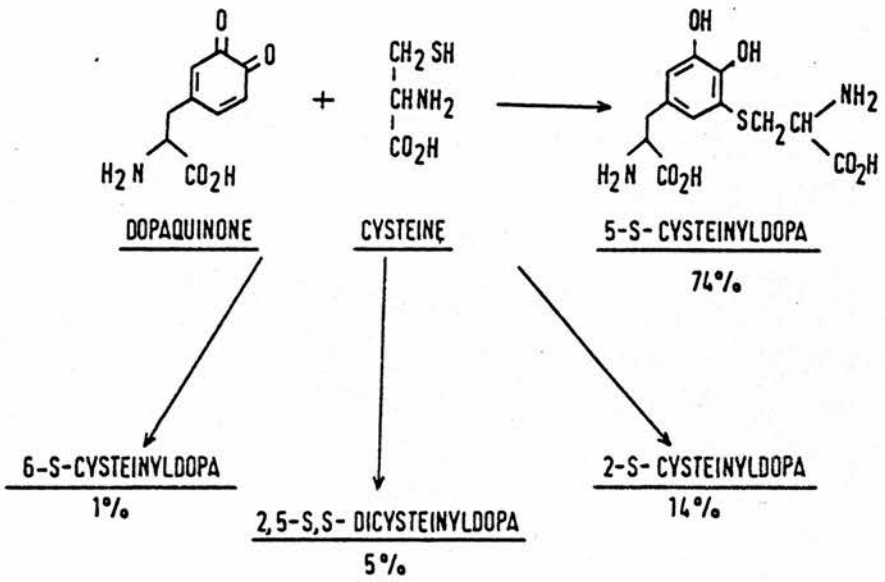

FIGURE 1.5 The cysteinyldopas produced on reaction of cysteine and dopaquinone

glutathionedopa, with γ-glutamyl transferase and a peptidase, to form cysteinyldopa (Agrup et al., 1975c) (Fig.1.6). Enzymatic oxidation of 5-S-CD in vitro in the presence of catalytic amounts of dopa has been shown to produce a red/brown polymer resembling naturally occurring phaeomelanins (Fattorusso et al., 1969). Further investigation has led to elucidation of part of the phaeomelanin and trichochrome synthetic pathways (Prota, 1980) (Fig.1.7). Enzymatic oxidation of 5-S-CD in the presence of dopa leads to the formation of 5-S-cysteinyldopaquinone. This then undergoes intramolecular condensation to produce the cyclic quinoneimine intermediate. Rearrangement of this molecule by a hydrogen shift results in the production of the phenolic isomer, or alternatively decarboxylation of the quinoneimine results in a further benzothiazine intermediate. The later steps in trichochrome synthesis are not as yet entirely clear, although it appears that the benzothiazine intermediates undergo oxidative dimerisation and decarboxylation to produce the various end products. It appears that the same benzothiazine intermediates are involved in the synthesis of the high molecular mass phaeomelanins, although the mechanism by which this occurs has not yet been characterised.

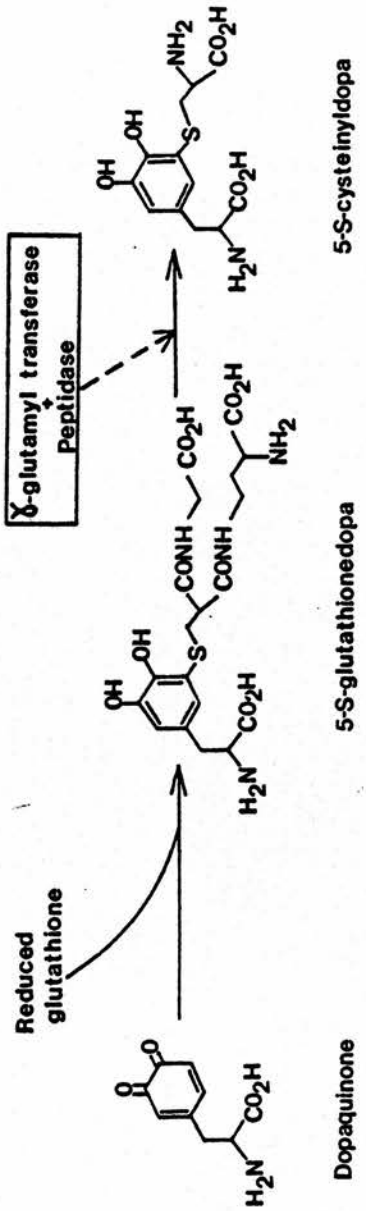
Reduced glutathione
γ-glutamyl transferase
Peptidase
Dopaquinone
5-S-glutathionedopa
5-S-cysteinyldopa

FIGURE 1.6 5-S-CD synthesis from glutathione and dopaquinone

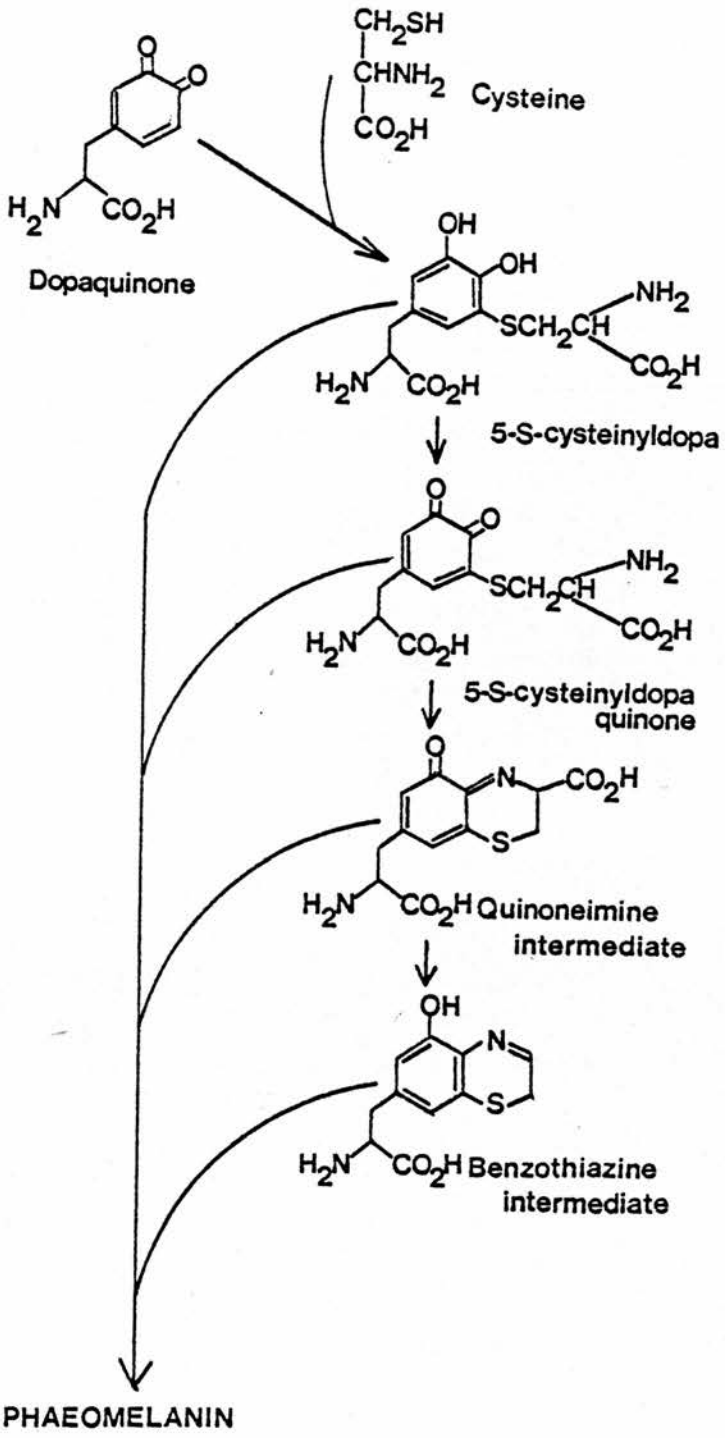

FIGURE 1.7 Phaeomelanin synthesis from dopaquinone and cysteine

Disorders of Pigmentation

Many disorders of pigmentation have been described. Some of these are acquired but many are genetic or congenital. The disorders can occur at several steps in the pigmentation process, including the differentiation of melanoblasts in the neural crest and a number of stages in the pathway of melanin synthesis. Two groups of pigmentary defect are of primary interest in these studies and will be described in more detail. These are oculocutaneous albinism and melanocytic tumours, including malignant melanoma.

Oculocutaneous albinism

Oculocutaneous albinism (OCA) is an inheritable disorder of the pigmentary system found throughout the animal kingdom. It is characterised by a decrease in, or a complete absence of, melanin in the skin, hair and eyes. In man at least four distinct varieties of albinism exist, these being tyrosinase-negative OCA (ty-neg OCA), tyrosinase-positive OCA (ty-pos OCA), yellow mutant albinism and Hermansky-Pudlack syndrome. All of these are inherited in an autosomal recessive fashion, and can be distinguished from one another on the basis of clinical and biochemical criteria (Bleehen and Ebling, 1979). The most severe form of OCA is ty-neg OCA, which may be considered the classic type of albinism in which the affected individuals have snow-white hair, pink-white skin, grey irides in tangential illumination, a prominent red reflex from a completely unmelanised fundus giving a 'pink eye' appearance, severe nystagmus and photophobia, and markedly decreased visual

acuity. Brown-pigmented naevi are absent from the skin, but small pink spots may be seen due to accumulations of non-pigmented naevus cells.

Electron microscopic examination of hair bulbs from ty-neg OCA patients shows melanocytes with stage I and II premelanosomes in which the unmelanised matrix is plainly visible (Birbeck and Barnicot, 1959; Witkop et al., 1970). Incubation in L-tyrosine or L-dopa does not appear to result in any increased pigmentation in the melanosomes, and there is no evidence of enzyme activity in the Golgi apparatus or endoplasmic reticulum (Kugelman and Van Scott, 1961; Witkop et al., 1970; Witkop, 1971). Premelanosomes do not appear to develop past Stage II. All available evidence points to a mutation at the tyrosinase locus such that no active tyrosinase is synthesised (Witkop et al., 1970; Witkop, 1971).

Patients with ty-pos OCA usually have some clinically detectable pigment, although as infants these individuals are phenotypically similar to those with ty-neg OCA. As they grow older, however, melanin synthesis increases, although it is still at a greatly reduced rate, and patients accumulate some pigment in the skin, hair and eyes. The hair may become tan, yellow or even red in colour. Photophobia and nystagmus may lessen with age as melanin accumulates in the choroid and retinal pigment epithelium. Visual acuity may also improve. Ultrastructural studies of hair bulbs from these patients reveal many stage I and II premelanosomes, and some pigmented stage III and IV melanosomes. Incubation of hair bulbs by the method of Kugelman and Van Scott (1961), results in

increased amounts of pigment discernible by light microscopy.

The basic defect in ty-pos OCA is unknown. Studies have suggested several possibilities which include a defect in the activation of tyrosinase, an intracellular inhibitor, or a defective feedback control mechanism (Pawelek et al., 1980).

The yellow-mutant form of albinism is common among the Amish people. Infants with this type of albinism resemble those with ty-neg OCA at birth, but during infancy the hair acquires a yellow tint which turns to red during childhood. Ocular symptoms improve with age, as in ty-pos OCA. Incubation of hairbulbs from these patients with tyrosine alone produces a negative reaction, whereas incubation with tyrosine and cysteine produces phaeomelanin (Bleehen and Ebling, 1979). These patients appear, therefore, to have a reduced ability to synthesize eumelanin.

Finally, the Hermansky-Pudlack syndrome is albinism associated with a storage disease of ceroid-like material in the reticuloendothelial system. In addition, a defect in platelet function occurs, often causing a severe bleeding diathesis (Witkop et al., 1973).

Melanocytic tumours

During childhood the normal ratio of melanocytes to basal epidermal cells (approximately 1:8) changes at certain sites, with the number of melanocytes becoming too great to be accommodated in their normal position. This proliferation of melanocytes leads to formation of a pigmented naevus. The type of naevus arising depends upon the position occupied by the proliferating melanocytes (Fig.1.8). When the melanocytes simply replace basal keratinocytes

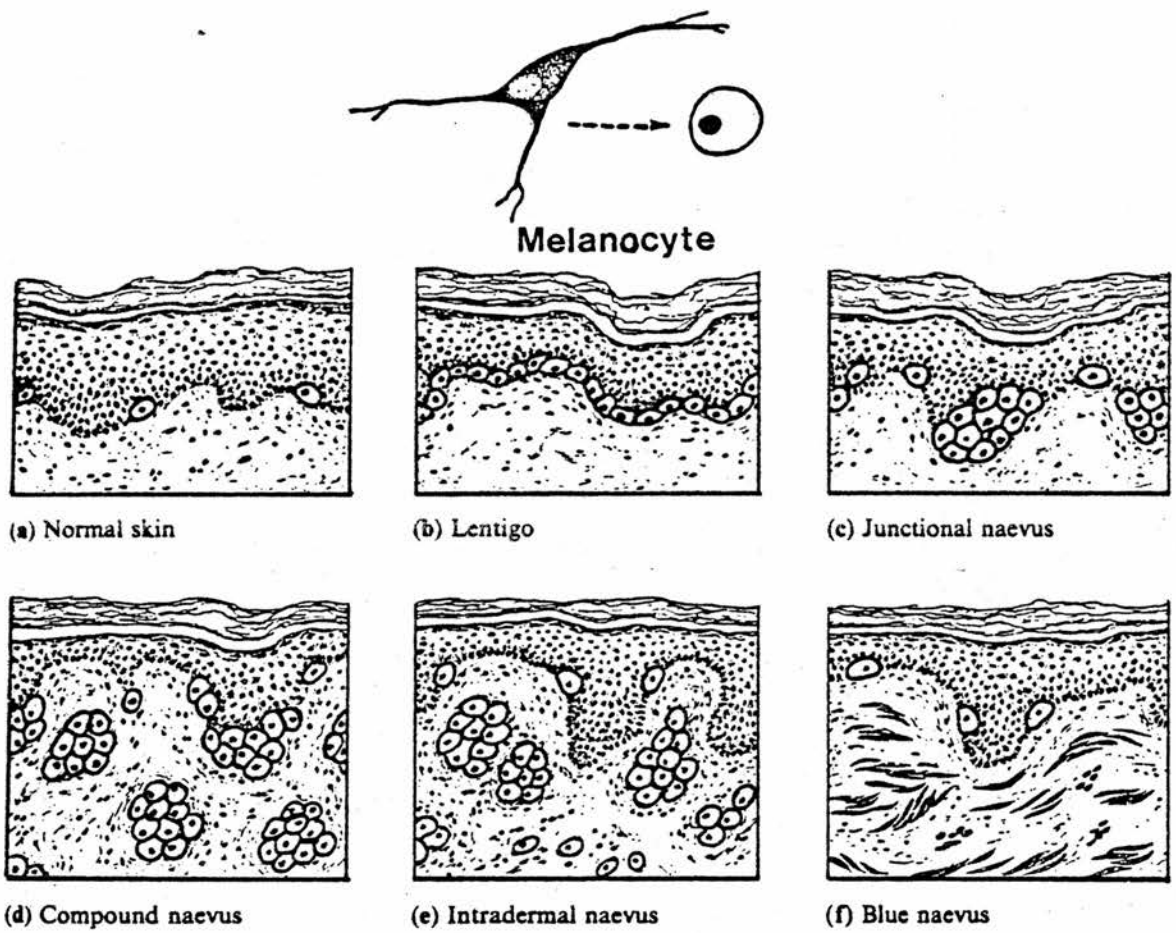

FIGURE 1.8 Various forms of pigmented naevus showing the position of the melanocytes
(From: Muir's Textbook of Pathology)

the lesion is known as a lentigo. A focal proliferation of melanocytes with formation of small nests of cells which bulge down into the dermis is known as a junctional naevus. A combination of this junctional activity with the movement of small packets of melanocytes completely into the dermis results in a lesion known as a compound pigmented naevus. The melanocytes within the dermis are termed naevus cells, and the deeper ones usually lose their ability to synthesize melanin. Frequently, in the early twenties, junctional activity ceases, the naevus cells in the dermis undergo further maturation and some fibrosis occurs, resulting in the production of an intradermal naevus which may remain as such for life. Although this maturation process is most often seen in the early twenties, similar stages of melanocytic proliferation may occur later in adult life as a result of exposure to UVR or hormonal changes. After puberty, any pigmented naevus with junctional activity has the potential to undergo malignant change. Histological features of a pigmented naevus which may point to early malignant change include a high mitotic rate, nuclear atypia, aberrant melanocytes in the superficial dermis, the presence of excessive melanin and a surrounding monohuclear inflammatory infiltrate.

The most malignant of skin tumours, malignant melanoma (MM) arises from the melanocyte. Some malignant melanomata develop from pre-existing pigmented naevi, whilst others arise de novo from apparently clinically normal skin. The tumour may be classified into four main forms, each characterised by distinctive clinical and histological features; lentigo maligna melanoma (LMM),

superficial spreading melanoma (SSM), acral lentiginous melanoma (ALM) and nodular melanoma (NM) (McGovern, 1970; Reed, 1976). The first three types are characterised by a preceding *in situ* horizontal growth phase from which an invasive melanoma develops. In NM there appears to be no horizontal phase. NM carries the worst prognosis of the various forms of MM, the best prognosis being in LMM. SSM and ALM give similar intermediate prognoses. SSM is the most commonly seen form of MM.

The prognosis for patients with primary MM is related to the depth of invasion of the primary tumour in the dermis at the time of its removal, and this may be assessed by the methods of Clark *et al.* (1969) or Breslow (1970). The method of Clark involves staging the lesion according to the level of invasion in the skin. *In situ* epidermal lesions are level I. Level II lesions are confined to the loose papillary dermis. In level III, the tumour forms a plaque at the junction of the loose papillary dermis and dense reticular dermis. In level IV there is invasion of the reticular dermis, and in level V there is involvement of the subcutaneous fat. The method of Breslow involves measurement of the maximal thickness of the lesion from the granular layer of the epidermis to the deepest point of invasion, by means of an ocular micrometer. Both of these methods are of value in assessing the prognosis in patients following surgical removal of a primary tumour. Other features which correlate with prognosis include histogenetic type of melanoma, as previously described, extent of ulceration, mitotic rate, and local invasion of lymphatics (Pondes *et al.*, 1981). The tumour

spreads both locally by direct invasion of the surrounding skin, by the lymphatics to local lymph nodes, and distally to other tissues via the lymphatics and blood. These are mainly liver, brain, lungs, bowel and bone marrow.

MM can be staged according to the extent of spread of the tumour. The most widely used staging system for MM involves three basic stages:

I - localised primary tumour

IA - localised recurrence (local satellites)

II - metastases to regional lymph nodes or intransit metastases

III - disseminated melanoma

(McNeer and Das Gupta, 1979).

Prognosis worsens as extent of spread of tumour increases.

Clinical features suggesting that a pigmented naevus or melanoma in situ has undergone malignant change include itching, alteration in colour, change in size, bleeding, ulceration and development of satellite lesions. Such change in character of a pigmented lesion in an adult warrant its excision and examination.

Treatment of primary MM at present is by wide surgical excision, perhaps in conjunction with removal of local lymph nodes.

As far as treatment of secondary MM is concerned, there are a number of choices depending on the circumstances of the individual patient. In asymptomatic patients, those who are terminally ill, or those of an advanced age, often no treatment is given. Surgical removal of the secondary tumour may be performed in situations in which the lesions are easily accessible and limited in amount, and

in patients in whom the operation can be performed safely. Irradiation is often used in patients with advanced melanoma at particular sites in the body, including bone and brain metastases and lesions located in the skin, subcutaneous tissues or lymph nodes. Effective chemotherapy for melanoma metastases is not currently available, with those drugs in current use providing a very poor response (Balch and Milton, 1985).

Recent epidemiological studies of MM in various parts of the world have demonstrated a rapid rise in the incidence of this tumour in white-skinned races (Mackie, 1983). The most rapid rate of increase, in the different areas studied, has been noted in Arizona, where the incidence of melanoma quadrupled in the period 1969-78 from 6·5 to 27·2 per 100000 population per year. It was noted that this increased incidence applied exclusively to those of Northern European descent. Studies from both Queensland, which has the highest recorded incidence of MM (32·7 per 100000 per year, in 1977), and North America, suggest that there is a higher incidence of melanoma in that proportion of the population who are of Celtic descent (Lane Brown, 1971). Indeed there is evidence that individuals from Scotland and Ireland who emigrated to the sunnier climates of Queensland and North America, and their descendants, have a higher incidence of cutaneous MM than other European immigrants. Study of the Scottish population in its native environment has indicated that the incidence of MM may also be increasing but had still only reached a level of approximately 5·1 per 100000 per year in 1979 (Mackie and Hunter, 1982). If the genotypes of the

affected Australians and the Scottish population are indeed similar, it would appear that environmental factors in Queensland have increased the current incidence of MM by a factor of 5 or 6.

There is considerable evidence that cumulative total lifetime sun exposure plays a significant role in the aetiology of LMM (McGovern et al., 1980). Intermittent sun exposure and episodes of burning may be more important causes of SSM and NM (Mackie and Aitchison, 1982).

The Scottish Melanoma Group was formed in 1978 with the specific aim of ensuring registration and proper documentation of all patients operated on for removal of a primary cutaneous melanoma, and following these patients from registration until death to look at changes in the patterns of the disease with time. Scotland is a particularly appropriate area for epidemiological study, for various reasons. The population is a remarkably static one; there is still considerable genetic homogeneity no longer found in many other parts of the world, especially in the USA, and, as it is a small country, information can be readily obtained from all parts.

In a study of South-East Scotland from 1961-76 a ratio of two females to every one male with MM in Scotland was found, in common with findings in other countries with a low incidence of the disease (Pondes et al., 1981). Again in common with other studies the most frequently found tumour was SSM on the lower limbs of females. The prognosis appeared to be better in females than in males. The thickness of the primary lesion was found to be the best prognostic index, with the 5 year survival rate ranging from 97 percent for

patients with tumours less than 0·5 mm deep, to 38 percent for those with tumours thicker than 3·5 mm. It was concluded that better survival figures were more likely to be achieved by earlier diagnosis and treatment of the tumour than by any other means, and that in general the most effective way of achieving this would be through better education of both doctors and the public, to teach them to recognise the early signs of possible malignant change.

Tumour Markers for Malignant Melanoma

The treatment of malignant disease has improved in the last few decades, an important part of the management being the monitoring of patients after primary diagnosis to assess prognosis and detect recurrence as early as possible. To this end much research is being carried out to find biochemical markers which will give the clinician information, above and beyond clinical signs and image intensifying procedures, about the state of the patient's disease.

The unique nature of the chemistry of the melanocyte suggests that there may be substances produced specifically by this cell which may be of use in the detection of MM. In the melanin synthesis pathway there are several intermediates which have been examined as possible tumour markers. The first two steps in melanin synthesis alone provide tyrosine, tyrosinase, dopa and dopaquinone as possibilities. Tyrosine is not found exclusively in melanocytes, being involved in general protein metabolism, so is of no value in this respect. Tyrosinase activity, on the other hand, has been detected in the serum of patients with MM in all but the earliest stages of the disease (Burnett, 1975). Nishioka et al., (1979) have found significantly higher tyrosinase activity in sera from MM patients compared with controls and patients with other malignancies. Chen and Chavin (1979), however, found increased serum tyrosinase activity in not only MM but also in many other malignant states, including breast cancer. It has been suggested that the apparent increased tyrosinase activity, measured as oxidation of substrate, may be due to increased non-specific

oxidation and may not reflect tyrosinase activity at all (Rorsman et al., 1983).

Dopa, like tyrosine, is also not found exclusively in melanocytes, being produced by the action of tyrosine hydroxylase in the nervous system and adrenals as well as by the action of tyrosinase in the melanocytes. Increased serum dopa levels have, however, been demonstrated in some patients with advanced metastatic melanoma (Hansson et al., 1978). A number of dopa metabolites have been studied, and reports have appeared of increased excretion of dopamine, dopac (dihydroxyphenylacetic acid) and homovanillic acid in the urine of some patients with MM. Another dopa metabolite, vanillactic acid, has also been observed in the urine of patients with melanoma and is considered by some to be a good indicator of metastatic disease (Duchon et al., 1967; Banda et al., 1980).

Dopaquinone, because of its highly reactive nature, is not readily detectable. One of the reactions which it may undergo, however, is intramolecular cyclization resulting in the indolic precursors of eumelanin. The Thormählen test, which is used to detect indole conjugates, has been used in the screening of urines from patients suspected of having metastatic melanoma (Duchon and Pechan, 1963). It appears that the finding of a continuously increasing excretion of these indolic compounds in the urine is always indicative of progression of the disease, irrespective of the absolute urinary levels found. Matous et al. (1980) found that in many cases an increase in Thormählen-positive compounds was seen prior to the diagnosis of metastatic disease on the basis

of clinical signs. The instability of these compounds, however, appears to limit the value of this method, and other workers have had less success with it (Haberman et al., 1976).

When cysteine is present in the melanocyte, dopaquinone is channelled into the phaeomelanin pathway by way of cysteinyldopa formation. It was the work of Rorsman and his group in Sweden which first demonstrated the presence of 5-S-CD and related compounds in melanomas and in the urine of patients with metastatic melanoma (Agrup et al., 1977a). The compounds which have been identified include 5-S, 2-S and 6-S-cysteinyldopa, 2,5-S, S-dicysteinyldopa, 4 O-methyl derivatives of 5-S-CD and 2-S-CD, and the trichochromes B and C (Agrup et al., 1976a; Prota et al., 1977). 5-S-CD has also been shown to be present in the urine of normal healthy subjects (Agrup et al., 1975b).

5-S-CD appears to be the most specific product of the melanocytes which has been studied, and as such has attracted attention as a marker for melanogenesis. Exposure to sunlight, a known stimulus to melanogenesis, has been shown to increase urinary excretion of 5-S-CD, thus demonstrating the ability of 5-S-CD to reflect the metabolic activity of the melanocyte (Rorsman et al., 1976; Nixon, 1978). It was this property which induced Rorsman's group to look at this melanin precursor as a possible marker for MM.

Methods that have been developed for the detection of 5-S-CD in urine, blood and tissues include ion-exchange chromatography combined with colorimetric detection, fluorimetry, gas chromatography - mass spectrometry and high-performance liquid chromatography with

electrochemical detection (Rorsman et al., 1973a,b; Banda et al., 1974; Agrup et al., 1976a; Blois and Banda, 1976; Banda et al., 1977; Hansson et al., 1978).

The fluorimetric assay developed by Rorsman et al. (1973a,b) has been used in several studies of urinary 5-S-CD levels. Studies of urinary excretion in normal healthy individuals and in patients with primary or secondary melanoma have provided evidence for the value of 5-S-CD in MM (Agrup et al., 1975a; Agrup et al., 1977a; Agrup et al., 1979a).

A five year study of 571 melanoma patients showed that 60 percent of patients with metastatic melanoma had raised urinary excretion of 5-S-CD, whereas 90 percent of those patients who did not develop secondaries had excretion rates within the reference range determined in healthy subjects (Agrup et al., 1979a). There were a number of patients, however, who were found to have normal urinary 5-S-CD excretion despite the development of metastatic disease. There did not appear to be any single explanation for this finding, although in some cases the secondary tumour tissue was found to be non-pigmented. However, these findings demonstrated that a normal urinary 5-S-CD level does not preclude the existence of metastatic disease. A number of the patients in this study were also found to have raised 5-S-CD levels whilst apparently remaining disease free, according to clinical criteria. However, these patients all developed metastases within 14 months of detection of a high urinary 5-S-CD level. There was a significant positive correlation between survival time and urinary 5-S-CD levels in this study;

only one patient with a high value survived for more than two years. In patients with no clinical signs of metastases who were followed up for two years, urinary 5-S-CD levels were similar to those in healthy individuals. The results of this study suggested, therefore, that measurement of urinary 5-S-CD in melanoma patients could be of value in determining the prognosis of a patient, and, in some cases in detecting metastatic disease earlier than the usual clinical investigations. However, the method did appear to have limitations, the most important being the percentage of patients who failed to develop raised urinary 5-S-CD levels despite the presence of metastatic disease. 5-S-CD levels were also shown to rise in response to exposure of the patient to sunlight, and in these cases the raised excretion of 5-S-CD was frequently as great as in patients with MM (Rorsman et al., 1976). For this reason the estimation of urinary 5-S-CD was performed, in Lund, only during the months of September to April, thereby missing out the effects of the summer sunshine.

The experience of Nixon (1978) in Brisbane also demonstrated the significance of UVR-induced increases in 5-S-CD excretion in a sunny climate. A study of 5-S-CD excretion in normal healthy subjects in response to a mild erythema dose of sun irradiation was carried out to determine whether it might be feasible to set 'normal limits' for 5-S-CD excretion under Australian conditions. However, increases in excretion of 5-S-CD in response to sunlight were found to be so great as to prevent the determination of useful limits which could be used to differentiate increased 5-S-CD excretion

after sun exposure from increased excretion in melanoma. The results, in fact, demonstrated that it was possible for a person of 'Celtic' complexion to achieve urinary 5-S-CD levels well above those of an individual with widespread metastatic melanoma, in response to frequent small doses of sun irradiation. It was concluded that this test could only be of use in a particularly sunny climate if patients could avoid exposure to sunlight for several weeks before the estimation of 5-S-CD. The results of the study of Rorsman et al., (1976), which led them to carry out 5-S-CD estimations only between September and April, demonstrated that even in very much less sunny climates the influence of UVR on 5-S-CD excretion may be quite considerable. This is obviously of major importance in the use of this test in MM.

In more recent years Rorsman's group have developed a high-performance liquid chromatography system for measurement of serum 5-S-CD (Hansson et al., 1978; Hansson et al., 1979a). This system is much more sensitive than the fluorimetric method used previously which was insufficiently sensitive to detect 5-S-CD in the serum of normal individuals. Only very limited reports of studies in serum have yet appeared in the literature, although there is some indication that serum 5-S-CD levels were higher in 7 melanoma patients than in healthy individuals (Hansson et al., 1978).

Aims

The aims of this work were to set up a method for the detection and measurement of 5-S-CD in plasma and to use it to study:-

1) plasma 5-S-CD levels in normal healthy individuals and,
2) the influence of the Edinburgh climate on these
3) the effect of artificial UVR on plasma 5-S-CD levels
4) the influence of skin and hair pigmentation, both normal and abnormal, on plasma 5-S-CD
5) the handling of 5-S-CD by the liver and kidneys
6) plasma 5-S-CD levels in patients with MM and other tumours, with particular emphasis on melanoma patients being followed up after surgical removal of a primary tumour.

MATERIALS AND METHODS

Materials

Chemicals

Chemicals and solvents were obtained from a variety of commercial sources and were of analytical grade or better. A full list of chemicals and suppliers is given in Appendix I.

Buffers

The composition of the buffers used in these studies is listed in Appendix II.

High-performance liquid chromatography

(a) General description

High-performance liquid chromatography (HPLC) is similar to gas chromatography (GC) in many respects, the important difference being the use of a liquid mobile phase in place of a gaseous one. HPLC complements GC in that it is of considerable use in the separation of compounds which cannot be easily volatilised, and is of particular value in the separation of compounds which are of high molecular mass, high polarity, or which are thermally unstable or tend to ionise in solution.

The increased viscosity of liquids as compared to gases means that in order to produce a similar flow through the system, the mobile phase in HPLC must be pumped at something like 20 to 100 times greater pressure than the mobile phase in GC. The high pressure in the system is produced by the pump, and pressures of 300 to 3000 psi are normally used.

The sample for analysis is small, and is injected directly by syringe or valve on to the column. Columns too are small, being typically 5mm in diameter and 10 to 25 cm in length. They are packed with highly specialised column packings with a uniform particle size of 5 to 10 μm.

Reverse-phase chromatography is the most commonly used HPLC technique. In this type of system the stationary phase is usually a hydrophobic bonded phase, such as octadecylsilane. The mobile phases are usually polar solvents such as water and mixtures of water and organic solvents. The retention of a solute is generally based upon its hydrophobic character, with polar solutes preferring

the polar mobile phase and eluting before the non-polar components which are retained by the hydrophobic stationary phase.

Reverse-phase ion-pair chromatography is of use in the separation of ionisable substances which are eluted rapidly and are not well separated by conventional reverse-phase systems. This type of chromatography uses the same mobile and stationary phases as regular reverse-phase techniques, but with the addition of an ion-pairing agent to the mobile phase. This agent is of opposite charge to the solutes of interest, and pairs with the solute ion to form an electrically neutral hydrophobic ion pair. This ion pair is retained by the stationary phase to a much greater extent than the solute ion alone, resulting in greater resolution of similar compounds. Ion-pairing has been employed in the studies described here, the agent being methanesulphonic acid.

The eluate from the column consists of a sequence of solute zones, separated with varying degrees of resolution, which pass through the detector. In an electrochemical detector, such as the one used in these studies, the solute zones pass into a very low volume thin-layer cell in which there is a carbon electrode held at a fixed potential. If the potential is greater than that required for electrolysis of the analyte, a measurable charge passes from the electrode to the analyte (or vice versa), with the resulting current being directly proportional to the concentration of solute present. Therefore, as the concentration of solute passing through the cell rises or falls the electrolysis current proportionately follows these changes. The current, as a function of time, is

amplified and sent to the recorder to yield the chromatogram.

(b) HPLC system used in these studies

The HPLC apparatus consisted of an Altex model 110A peristaltic pump (Altex Scientific Inc., Berkeley, Calif., USA), a Rheodyne model 7125 sample injector with a 50 µl sample loop (Rheodyne Inc., Cotati, Calif., USA), and a BAS amperometric detector, model LC-4A with glassy carbon electrode (Bioanalytical Systems Inc., West Lafayette, Ind., USA). The detector cell was operated at +0·75V versus an Ag/AgCl reference electrode. The column was stainless steel (250 x 5 mm) packed with ODS-hypersil, C18 reverse-phase packing material, 5µm particle size (Shandon Southern Products, Ltd., Runcorn, Cheshire, UK). The column was packed by an upward slurry technique. The system was run at ambient temperature (20°C). The mobile phase consisted of methanesulphonic acid, 60 mmol/l, as ion pairing agent, and orthophosphoric acid, 30 mmol/l, in water purified by the Elgastat Spectrum reverse osmosis system (Elga Group, Lane End, Bucks., UK). The mobile phase was thoroughly degassed under vacuum before use.

The HPLC system is shown diagramatically in Fig.2.1.

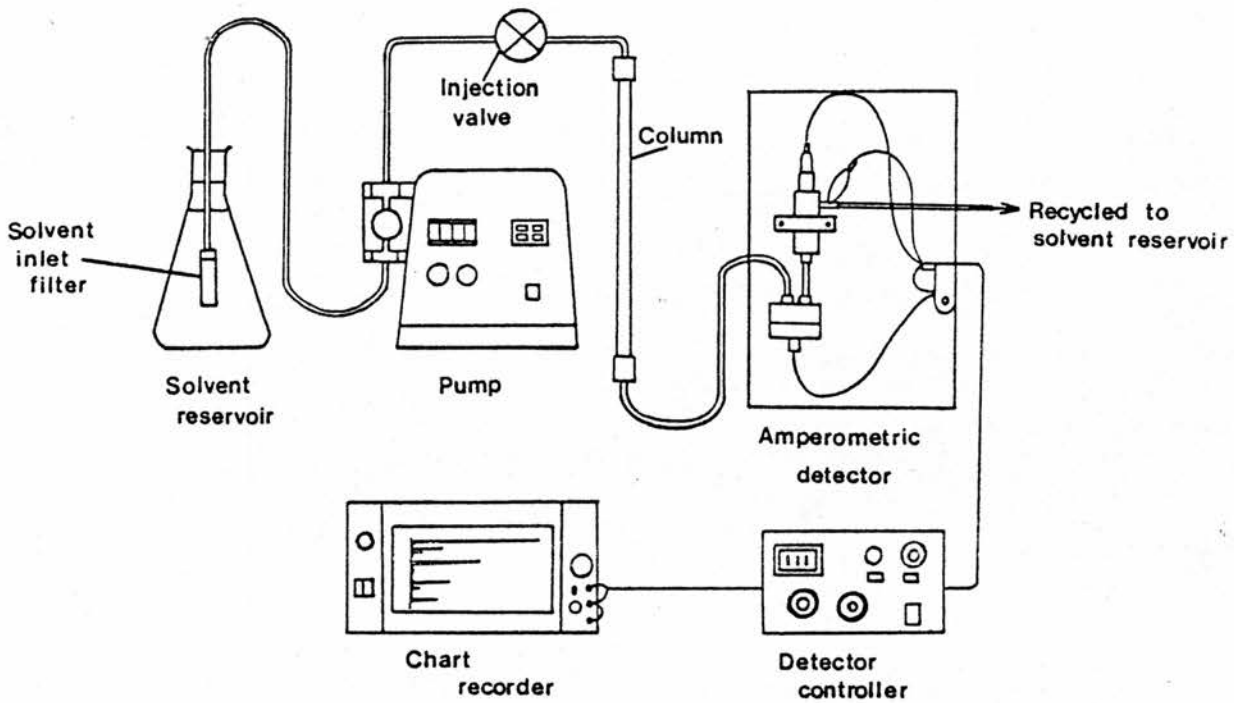

FIGURE 2.1 The HPLC system used in these studies

Preparation of standards

(a) Synthesis

5-S-L-cysteinyl-L-dopa and 5-S-D-cysteinyl-L-dopa were prepared by the reaction of L-dopa with L-cysteine or D-cysteine, respectively, in the presence of mushroom tyrosinase. The source of these chemicals is given in Appendix I.

10mg L-dopa and 12mg cysteine (D or L) were dissolved in 6ml Sorensens phosphate buffer, 0·5M, pH 6·5 (Appendix II). 3·75mg tyrosinase was dissolved in 2ml of the same buffer. The two solutions were mixed, and kept stirring vigorously for 1h at room temperature (20°C). The time course of the reaction, in terms of appearance of 5-S-CD and disappearance of dopa, is shown in Fig.2.2.

The reaction was stopped after 1h by the addition of 6M HCl to take the solution to pH 1. The final product of the reaction between L-dopa and L-cysteine, when analysed using HPLC, was seen to contain 6 peaks, the largest of which, with the longest retention time was found to co-chromatograph with authentic 5-S-L-cysteinyl-L-dopa (kindly donated by Professor Hans Rorsman). Fig.2.3 shows the chromatogram of the final reaction product and identifies the peak thought to represent 5-S-CD (peak X).

The final product of the reaction between L-dopa and D-cysteine was similar to that in Fig.2.3, on HPLC analysis.

(b) Purification of reaction products

The reaction products were separated using ion-exchange chromatography. A Quickfit column (1 x 25 cm) was packed with AG50W-X8 ion exchange resin (200-400 mesh size, H^+ form). The

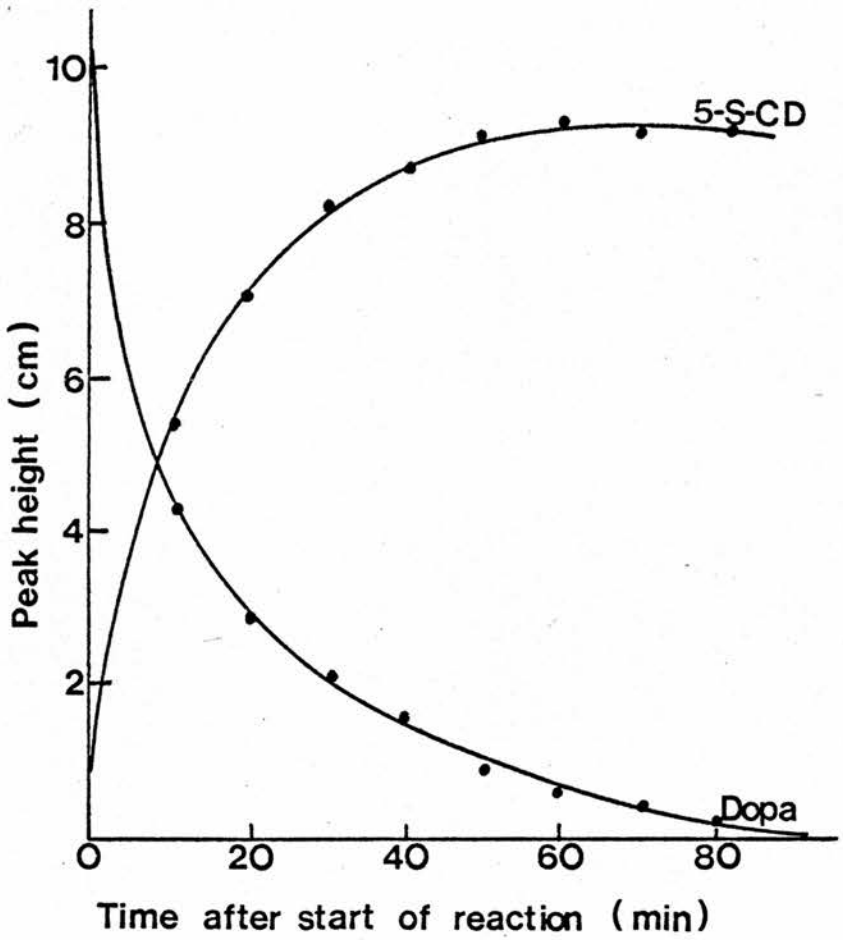

FIGURE 2.2 Time course of the reaction between cysteine and dopa in the presence of tyrosinase, in phosphate buffer 0·5M, pH 6·5 at 20°C

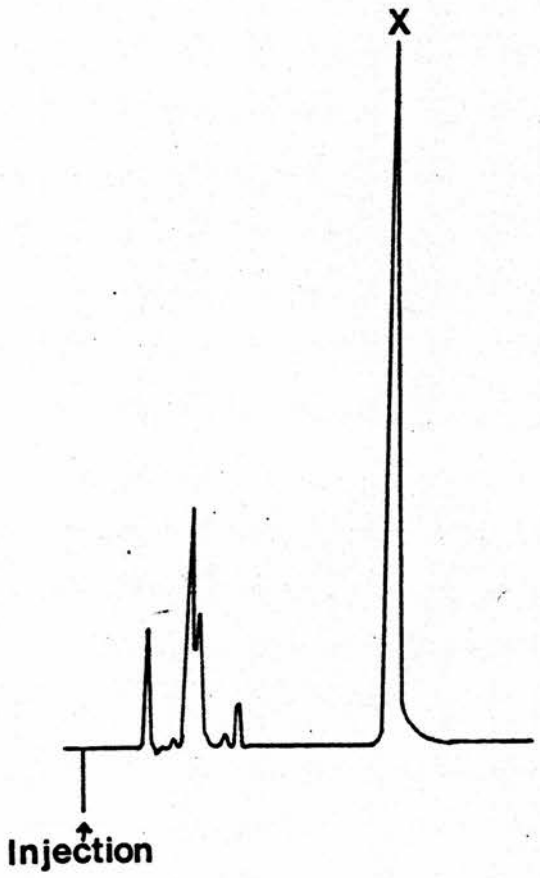

FIGURE 2.3 HPLC chromatogram of final product of reaction between cysteine and dopa in the presence of tyrosinase
Peak X thought to represent 5-S-CD

resin was equilibrated with 0·1M HCl. The final reaction mixture was applied to the column and washed through with 20ml of 0·5M HCl at a rate of 30-40 ml/h. Elution was performed with 3M HCl at a rate of 30-40 ml/h. 5ml fractions were collected from the column and analysed by HPLC. The ion-exchange profile for the reaction product of L-dopa and L-cysteine is given in Fig.2.4, where fraction number is plotted against the height of the 6 product peaks seen on HPLC. A similar ion-exchange profile was obtained for the reaction product of L-dopa and D-cysteine. In both cases, the fractions containing the largest amounts of peak X, thought to be the 5-S-CD product, were pooled and taken to dryness at 37°C in a rotary evaporator. Complete dryness was achieved under vacuum in the presence of solid NaOH. The product was redissolved in 3ml of 2M HCl.

A second ion-exchange step was performed to further separate the 5-S-CD product from contaminants. A Quickfit column (1 x 20 cm) was packed with AG50W-X8 resin (200-400 mesh size, H^+form) and the resin equilibrated with 0·1M HCl. The redissolved product of the first ion-exchange step was applied to the column. The column was eluted with 2M HCl at a flow rate of 30-40 ml/h. 5ml fractions were collected and analysed by HPLC. Fig.2.5 shows the fraction number plotted against the height of peak X in each fraction. This peak was not contaminated by measurable amounts of any of the other reaction products, which were seen to elute in earlier fractions. Again, the ion-exchange profile for the D-product was similar. The peak fractions in each case were pooled, and 2ml portions of the pooled product stored at -20°C until used.

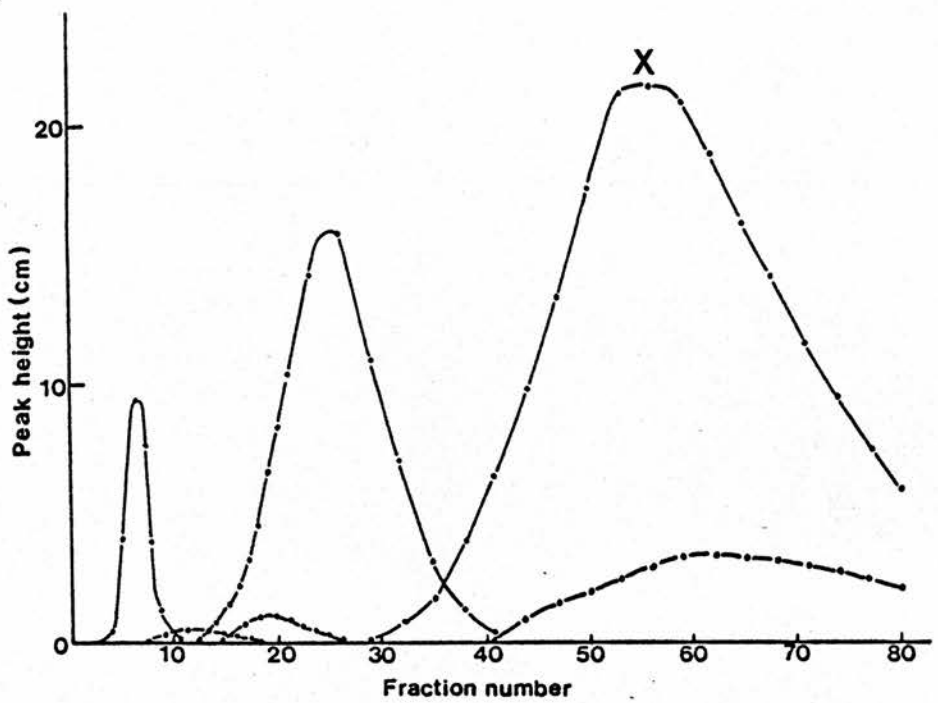

FIGURE 2.4 Ion-exchange profile of product of reaction between L-cysteine and L-dopa from a 25cm column of AG50W-X8 ion-exchange resin eluted with 3M HCl at a flow rate of 40 ml/h
Peak X thought to represent 5-S-CD

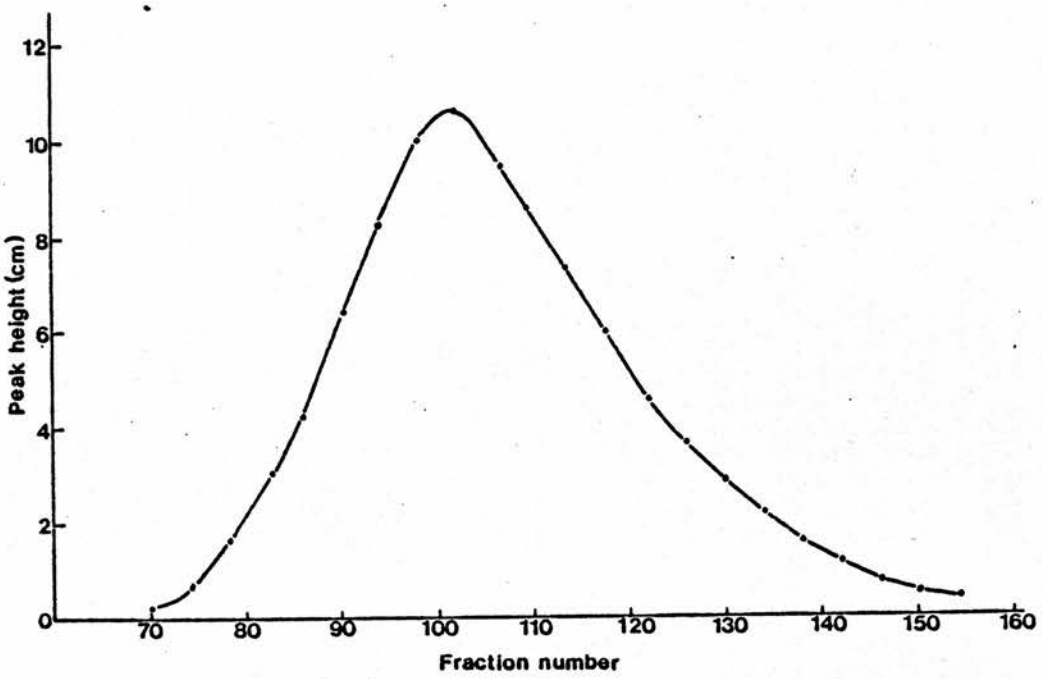

FIGURE 2.5 Ion-exchange profile of pooled fractions 45 - 70 (from ion-exchange step 1) from a 20cm column of AG50W-X8 ion-exchange resin eluted with 2M HCl at a flow rate of 40 ml/h, showing presence of one product only between fractions 70 and 160 (peak X)

(c) Identification of products

The final products of these purifications were subjected to a number of procedures in which their properties were compared to those of authentic 5-S-L-cysteinyl-L-dopa (authentic 5-S-D-cysteinyl-L-dopa was not available).

(i) Fluorimetry

The fluorimetric assay used was that developed by Rorsman et al. (1973a) for the measurement of 5-S-CD. The measurements were performed on an Aminco SPF 125S spectrophotofluorimeter (American Instrument Co., Maryland, USA). The purified reaction product was diluted 1 in 100 with distilled water and taken through the fluorimetric procedure. Emission and excitation spectra were obtained for each product, and are shown in Fig.2.6. Maximum excitation was seen at 315-320 nm for both products, and maximum emission occurred at 415 nm. Comparison with authentic 5-S-L-cysteinyl-L-dopa showed similar excitation and emission spectra.

Fluorimetric analysis of the reaction substrates, cysteine and dopa, showed minimal fluorescence under the conditions of study, and analysis of sequential samples from the reaction mixture during the course of the reaction demonstrated an increase in fluorescence with time as 5-S-CD was formed.

(ii) Ultraviolet absorbance

The ultraviolet absorption spectra of the reaction products were examined on a Pye Unicam SP8-100 spectrophotometer (Pye Unicam, Cambridge, UK). The spectra obtained are shown in Fig.2.7. Second order derivatives of the absorption curves were also obtained,

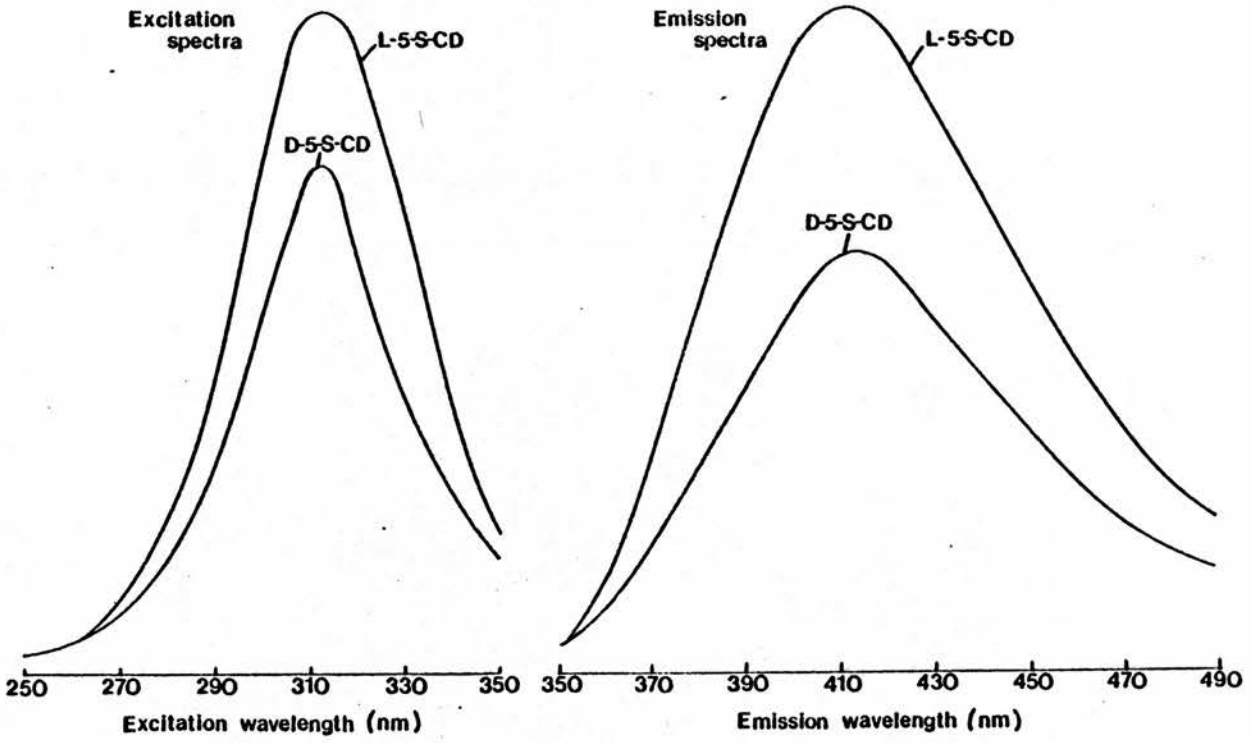

FIGURE 2.6 Fluorimetric excitation and emission spectra of purified products of reaction between L- or D-cysteine and L-dopa, thought to be the L and D forms of 5-S-CD

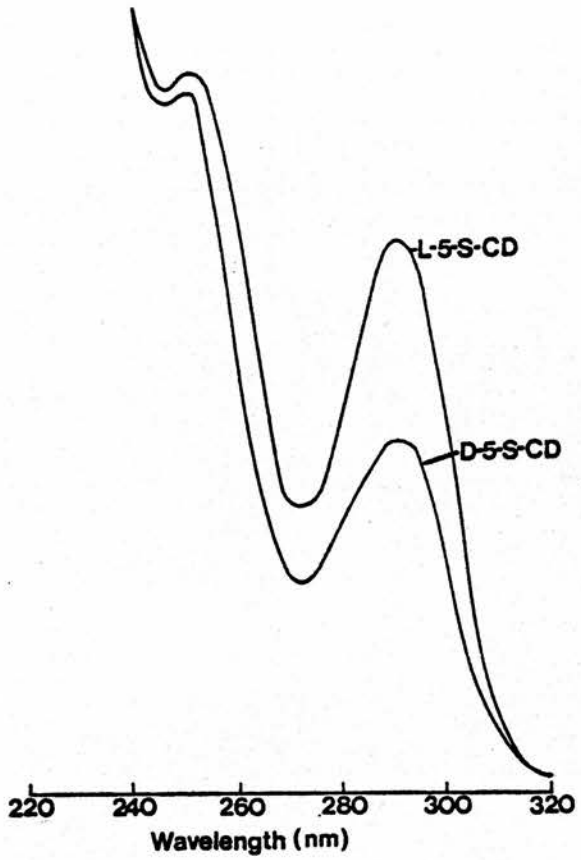

FIGURE 2.7 UV absorption spectra of purified products of reaction between L- or D-cysteine and L-dopa, thought to represent the L and D forms of 5-S-CD

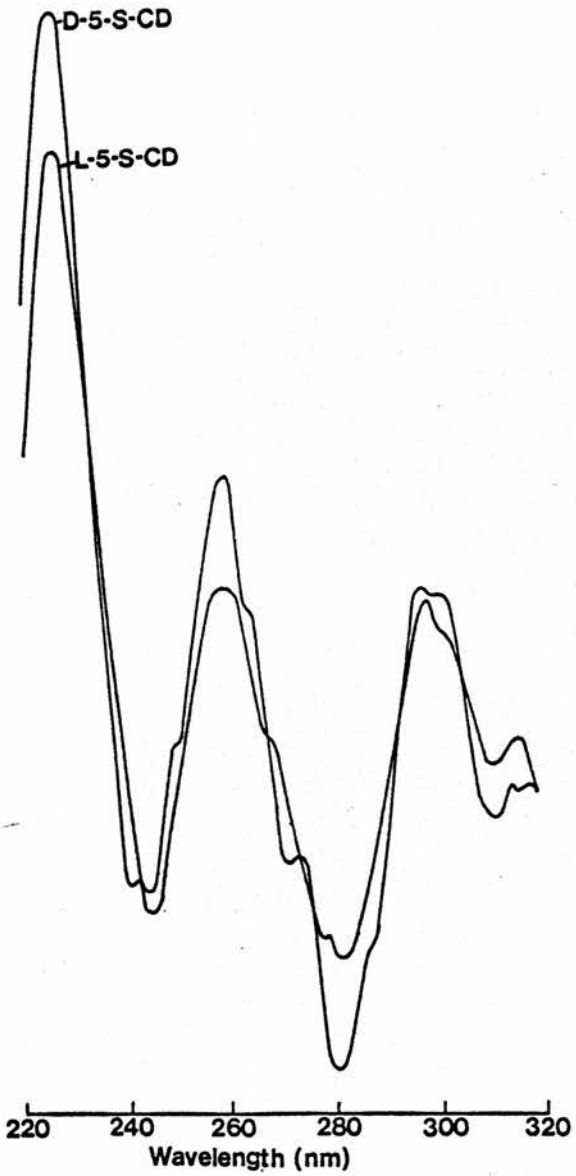

FIGURE 2.8 Second order derivatives of UV absorption spectra of the purified reaction products thought to be the L and D forms of 5-S-CD

and are shown in Fig.2.8. The absorption characteristics of the reaction products were very similar to those obtained for authentic 5-S-L-cysteinyl-L-dopa, and to those published by Agrup et al. (1976b). Strong absorption maxima were observed at 292 and 255 nm for both products.

The concentrations of the two cysteinyldopas in the final solutions could be calculated from the absorbance data, using the equation $c = A/\varepsilon \times 1$, where c is the concentration (mol/l), A is the absorbance at 292 nm, ε is the molar extinction coefficient (ε = 2800, Agrup et al., 1983), and 1 is the light path length (cm). The concentrations of the two products were found to be 50µg/ml and 40µg/ml for the L form and D form, respectively.

(iii) HPLC

The HPLC system described on page 42 was used in the analysis of the purified reaction products. The chromatograms obtained are shown in Fig.2.9. In each case there was one peak with virtually undetectable levels of contamination from other products. The product thought to be 5-S-L-cysteinyl-L-dopa was found to co-chromatograph with authentic standard material. The product thought to be 5-S-D-cysteinyl-L-dopa was found to be well separated from the L form in the solvent system used, as reported by Agrup et al. (1983).

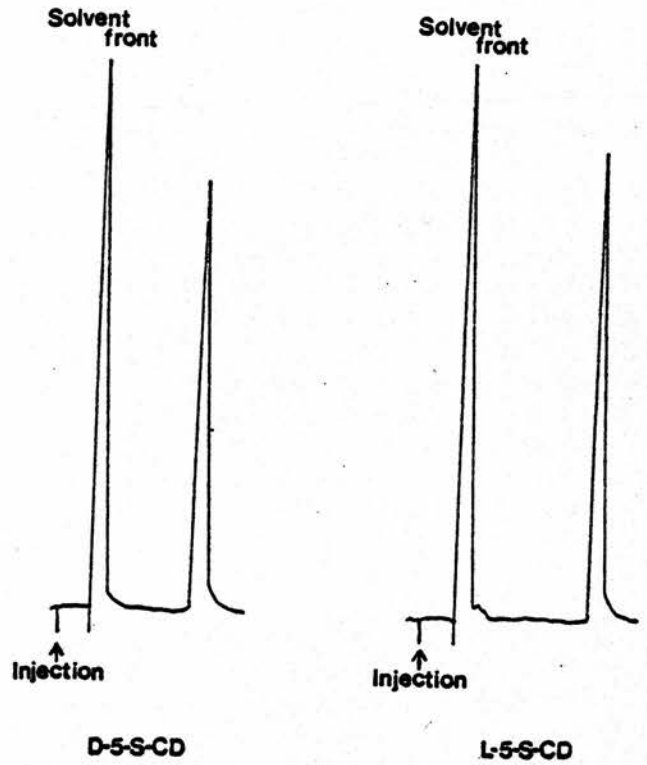

FIGURE 2.9 HPLC chromatograms of purified products of reaction between L- or D-cysteine and L-dopa, thought to be the L and D forms of 5-S-CD

Preparation of plasma samples

(a) Activation of aluminium oxide

100g aluminium oxide (Al_2O_3) was heated in 500ml 2M HCl at 90°C for 45 min, with continuous and rapid stirring. The Al_2O_3 was allowed to settle and the supernatant removed and discarded. The Al_2O_3 was washed twice with fresh 250ml portions of 2M HCl at 70°C for 15 min, and the supernatant discarded each time. The Al_2O_3 was washed finally with 500ml 2M HCl at 50°C for 10 min. The supernatant was discarded and the Al_2O_3 washed approximately 20 times with fresh 200ml portions of distilled water, until a pH of 3·4 was reached. The Al_2O_3 was transferred to an evaporating dish, heated at 120°C for 1h and 200°C for 2h. The activated Al_2O_3 was stored in a stoppered glass container.

(b) Extraction of 5-S-CD from plasma

Blood samples were taken into lithium-heparin tubes containing dithiothreitol (final concentration 5mM) as preservative. Samples were centrifuged at 2500 g for 10 min at 20°C, and the plasma separated off using a Pasteur pipette. 2ml plasma was used for the assay of 5-S-CD. The plasma was placed in a tube with internal standard (5-S-D-cysteinyl-L-dopa) and mixed well. 50µl reduced glutathione solution (0·05M) was added with 50µl ethylenediaminetetraacetic acid solution (EDTA:0·3M, pH 7·0) and 20mg activated Al_2O_3, the tube contents mixed, and 20µl Tris buffer (1M, pH 8·6) added during mixing. The tubes were placed in a vortex mixer, and the tube contents mixed vigorously for 15 min. The tubes were centrifuged at 3000g for 5 min at 20°C, to take down the Al_2O_3 and

the supernatant removed and discarded. The Al_2O_3 was washed three times with 2·5ml EDTA solution (0·003M, pH 8·0). After each wash, the tubes were centrifuged at 3000g for 5 min at 20°C, and the supernatant discarded. 150µl perchloric acid (0·2M) was added to the Al_2O_3 and the tubes mixed vigorously in a vortex mixer for 1 min. After a final certrifugation at 3000g for 5 min at 20°C, 50µl of the supernatant was taken for HPLC analysis.

(c) Recovery of cysteinyldopa from plasma

A calibration curve was prepared by injecting known amounts of standard material, both 5-S-L-cysteinyl-L-dopa (L-5-S-CD) and 5-S-D-cysteinyl-L-dopa (D-5-S-CD), made up in 0·2M perchloric acid, directly into the HPLC system and measuring the resulting peak heights.

Duplicate plasma samples were prepared, each 2ml in volume. To one of these was added a known mass of each 5-S-CD standard. Both samples were taken through the extraction procedure and analysed by HPLC. The peak heights of the two 5-S-CDs in each sample were noted. Percentage recovery of each 5-S-CD was calculated as follows, if (a) is the sample with no added standards, and (b) is the sample with added standards.

Peak height of L-5-S-CD in (a) is x

Peak height of L-5-S-CD in (b) is y

Peak height representing added L-5-S-CD is (y - x)

Mass of L-5-S-CD corresponding to a peak height of (y - x) was read from the calibration curve, and from this the mass of L-5-S-CD in the extract was calculated. This was compared with

the mass of L-5-S-CD added to the plasma prior to extraction, and the ratio of the two values gave the percentage recovery of L-5-S-CD from the procedure. The recovery of D-5-S-CD was calculated in the same manner, although there was, of course, no D-5-S-CD present in the plasma to which no standard had been added.

Four pairs of duplicates were prepared for calculation of recovery of L-5-S-CD and D-5-S-CD, and the results are given in Table 2.1.

(d) Preservation and storage of blood samples

Storage of whole blood, serum and plasma

60ml blood was taken from a volunteer and placed in tubes as follows:- 10ml in a plain glass tube, and the remaining 50ml in 5 10ml plastic lithium-heparin coated tubes, for the following studies.

(i) Comparison of serum and plasma in 5-S-CD estimation

One blood sample in a glass tube, and one in a plastic lithium-heparin tube were centrifuged at 2500g for 15 min at 20°C. Two portions, each of 2ml, of plasma and serum were obtained, extracted and analysed.

(ii) Overnight storage of whole blood and plasma

Two samples in lithium-heparin tubes were taken. One was stored as whole blood overnight at 4°C, whilst the other was centrifuged at 2500g for 15 min at 20°C, and the plasma obtained stored at 4°C overnight. The blood sample was centrifuged, under the same conditions, the following day, and the plasma removed. Two portions, each of 2ml, of each plasma, were extracted and

Table 2.1 Recovery of L-5-S-CD and D-5-S-CD from plasma

	Percentage recovery	Mean percentage recovery
L-5-S-CD	50, 47, 50, 54	50
D-5-S-CD	51, 48, 57, 60	54

analysed.

(iii) Effect of antioxidant on 5-S-CD levels in stored plasma

Two 10ml blood samples in lithium-heparin tubes had dithiothreitol added to final concentrations of 5 and 25 mmol/l. The samples were centrifuged at 2500g for 15 min at 20°C, and the plasma removed from each and stored overnight at 4°C. Two portions, each of 2ml, of each sample were extracted and analysed.

The results of these studies are given in Table 2.2. Extraction of 5-S-CD from plasma appeared to be slightly more efficient than extraction from serum. In addition, the plasma samples were easier to prepare, both in terms of time required to obtain plasma from the samples, and in the use of plastic tubes which were less readily broken during centrifugation. All further studies were therefore carried out using plasma.

The mass of 5-S-CD recovered from samples stored overnight did not seem to be affected by whether the samples were stored in the form of whole blood or plasma or whether antioxidant was added to the sample prior to storage for this short period of time.

Further studies of plasma storage

A 50ml pool of plasma was prepared, divided into 2ml portions, and used in the following studies:-

(i) Two 2ml portions were immediately extracted and analysed

(ii) Two 2 ml portions were stored for 3 days

(a) at 20°C

(b) at 4°C

(c) at -20°C

(d) with 5mM dithiothreitol at -20°C

Table 2.2 Storage of whole blood, serum and plasma

Sample	5-S-CD conc'n (nmol/l)	Mean 5-S-CD conc'n (nmol/l)
(i) Plasma	47, 49	48
Serum	40, 44	42
(ii) Whole blood	48, 50	49
Plasma	47, 48	47·5
(iii) Plasma plus 5mM dithiothreitol	49, 51	50
Plasma plus 25mM dithiothreitol	48, 49	48·5

(iii) Two 2ml portions were stored for 1 week

(a) at 20°C

(b) with 5mM dithiothreitol at 20°C

(iv) As for (iii), but storage for 2 weeks

(v) As for (iii), but storage for 4 weeks

The results of these studies are given in Table 2.3. 5-S-CD in plasma was stable for up to 4 weeks when dithiothreitol was added prior to storage at -20°C.

Table 2.3 Further studies of plasma storage

Sample	5-S-CD concn (nmol/l)	Mean 5-S-CD concn (nmol/l)
(i) Plasma	32, 35	33·5
(ii) (a) Plasma	29, 28	28·5
(b) Plasma	31, 33	32
(c) Plasma	29, 30	29·5
(d) Plasma plus dithiothreitol	31, 33	32
(iii) (a) Plasma	26, 27	26·5
(b) Plasma plus dithiothreitol	32, 33	32·5
(iv) (a) Plasma	25, 28	26·5
(b) Plasma plus dithiothreitol	32, 34	33
(v) (a) Plasma	28, 29	28·5
(b) Plasma plus dithiothreitol	33, 35	34

Identification of cysteinyldopas in plasma

(a) Co-chromatography with standards

Plasma extracts were subjected to HPLC under varying chromatographic conditions. Plasma extracts with added 5-S-L-cysteinyl-L-dopa were also analysed. Fig.2.10 shows the effect of varying the pH of the mobile phase on the retention of the plasma peak thought to represent 5-S-CD. The change in retention of authentic 5-S-CD in response to changing pH was seen to parallel the change in retention of the plasma peak.

Fig.2.11 shows the effect of varying the concentration of ion-pairing agent in the mobile phase on retention of both plasma and standard 5-S-CD peaks. Again the changes are seen to run in parallel.

Fig. 2.12 shows examples of the co-chromatography of authentic 5-S-CD with the plasma peak thought to represent 5-S-CD.

These studies lend support to the provisional identification of the peak as 5-S-CD.

(b) Oxidation properties

As the electrical potential applied across a solution undergoing analysis by electrochemical means is changed, the size of the resulting chromatographic peaks, representing the analytes present in the solution, will also vary, and the way in which they vary is characteristic of each particular analyte, and dependent on its oxidation properties. A hydrodynamic voltammogram can be constructed for each analyte which demonstrates the way in which chromatographic peak height varies with changing potential. The hydrodynamic voltammogram for 5-S-CD is shown in Fig.2.13. This figure also

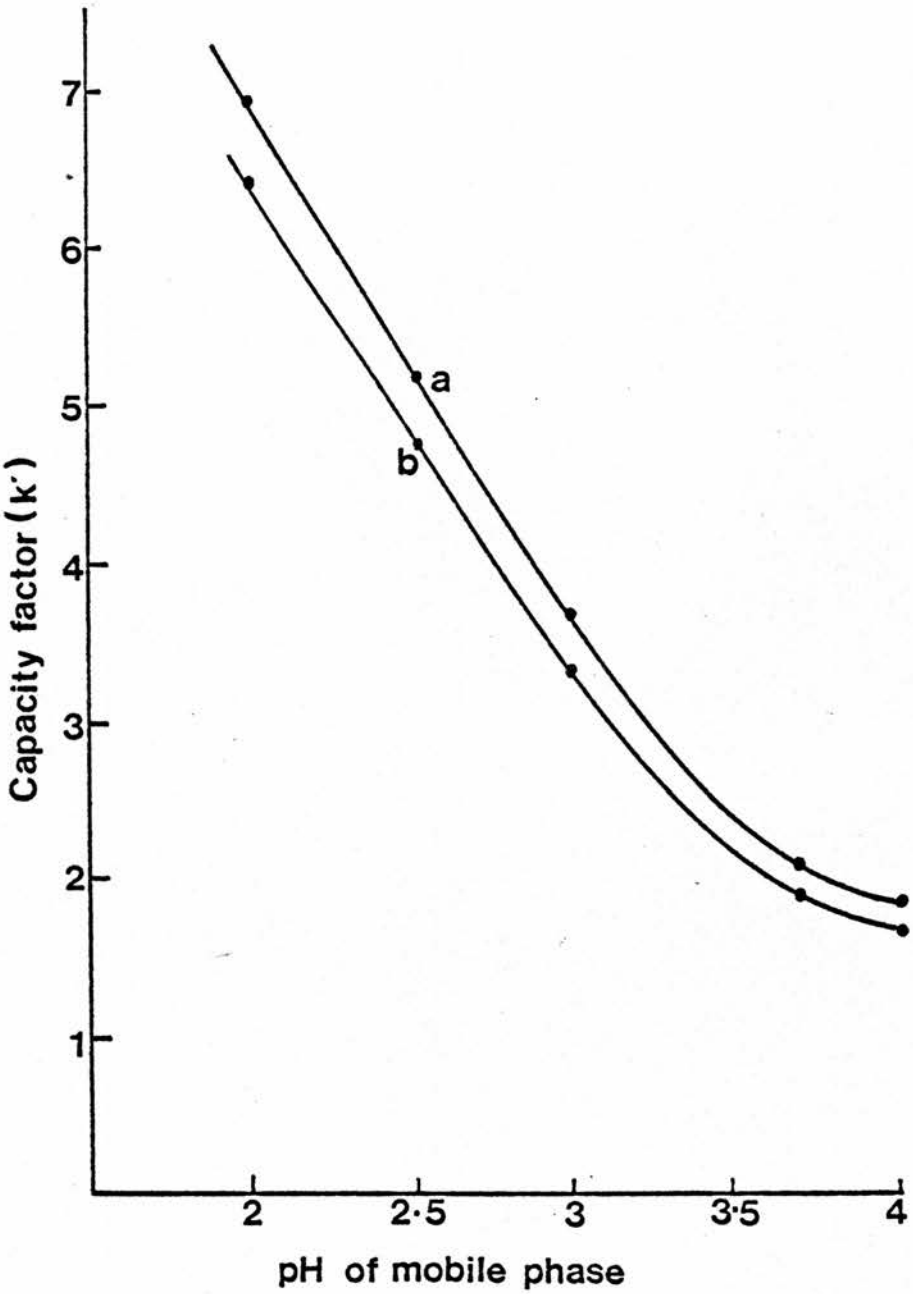

FIGURE 2.10 The effect of pH of the mobile phase on retention of 5-S-CD on a HPLC column, plotted as pH versus capacity factor (k')

$k' = \frac{V_r - V_o}{V_r}$, where V_r = elution volume

V_o = void volume

(a) authentic 5-S-CD standard

(b) plasma peak X thought to represent 5-S-CD

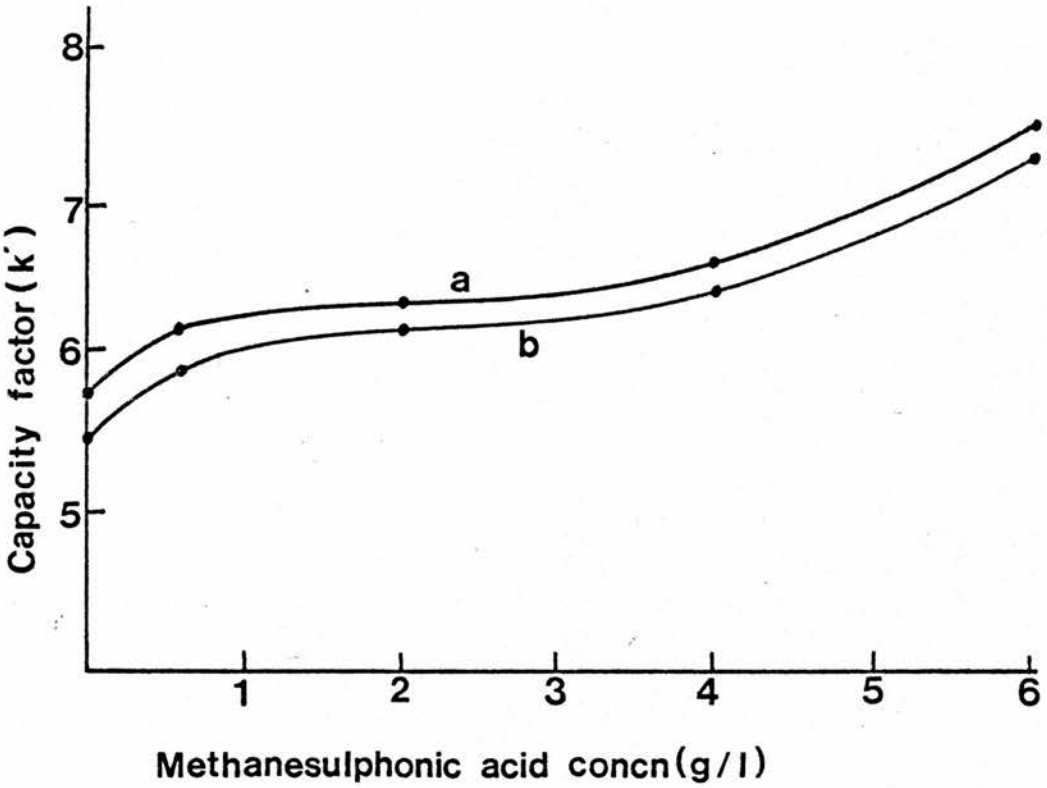

FIGURE 2.11 The effect of ion-pairing agent concentration of the mobile phase on retention of 5-S-CD on a HPLC column, plotted as concentration versus capacity factor (k')

(a) authentic 5-S-CD standard

(b) plasma peak X thought to represent 5-S-CD

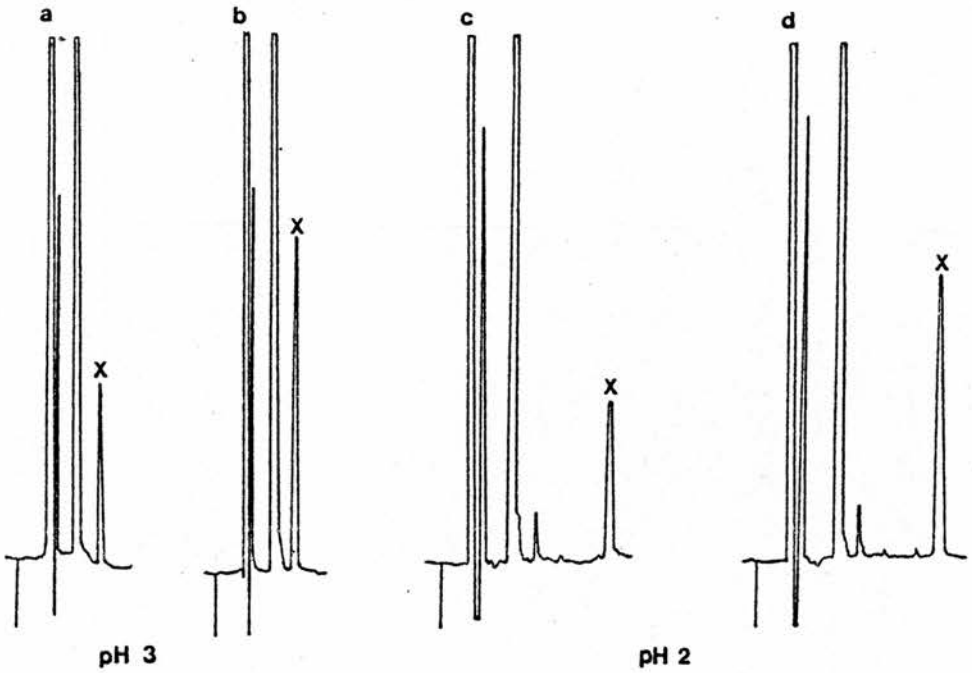

FIGURE 2.12 Co-chromatography of plasma extract and standard 5-S-CD in a HPLC system with mobile phase of differing pH

a and c - plasma extract

b and d - plasma extract plus standard 5-S-CD

X = 5-S-CD peak

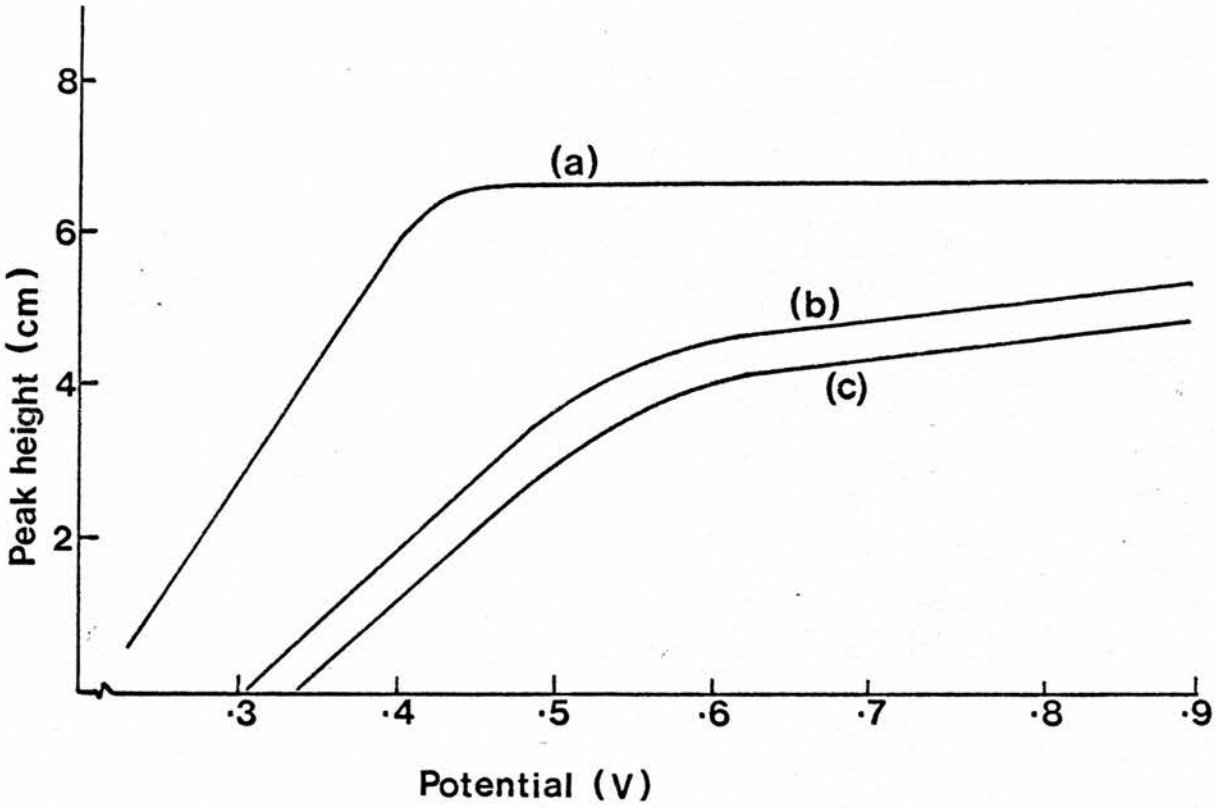

FIGURE 2.13 Hydrodynamic voltammogram (peak height on HPLC chromatogram versus electrical potential applied across solution by amperometric detector) for standard 5-S-CD and plasma peak X

(a) standard 5-S-CD in water

(b) standard 5-S-CD in plasma

(c) plasma peak X

shows the voltammogram obtained for 5-S-CD added to plasma, and it can be seen that it is not identical to that obtained for 5-S-CD in water. This is probably due to interference from other oxidisable substances present in the plasma. The figure also shows the voltammogram for the chromatographic peak thought to represent naturally-occurring plasma 5-S-CD, and this is seen to be very similar to that obtained for 5-S-CD added to plasma, further supporting the identity of this peak as 5-S-CD.

(c) Identification of other cysteinyldopas in plasma by retention times

The identities of some of the reaction products of (2) (a), other than 5-S-CD were deduced on the basis of elution order from ion-exchange and HPLC columns, and the relative amounts of each product formed, as compared with the findings of Ito and Prota (1977). Fig. 2.14 shows the chromatogram of the final reaction product and the likely identities of a number of the peaks.

Although this identification was largely speculative, the peaks which were seen in plasma and seemed to correspond to the identified reaction products were those which, in addition to 5-S-CD, were seen to be considerably larger in plasma from some patients with metastatic melanoma or in other situations in which elevated 5-S-CD levels were found. Fig.2.15 demonstrates the increase in size of a number of the peaks, seen in plasma from a patient with metastatic melanoma.

Calibration and routine use of the plasma 5-S-CD assay

(a) Construction of calibration curves

Dilutions of 5-S-CD standard in water and plasma were prepared

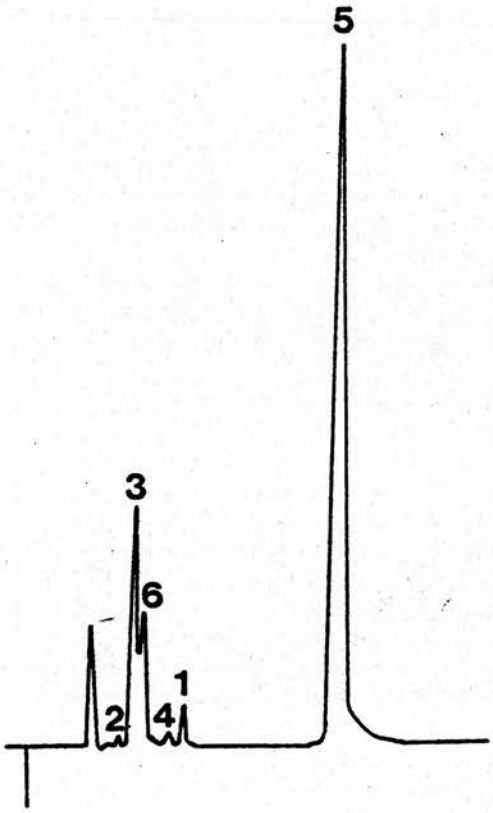

FIGURE 2.14 HPLC chromatogram of product of reaction between cysteine and dopa in the presence of tyrosinase giving suggested peak identifications

1 = 6-S-CD

3 = 2-S-CD

5 = 5-S-CD

6 = 2,5-S,S-diCD

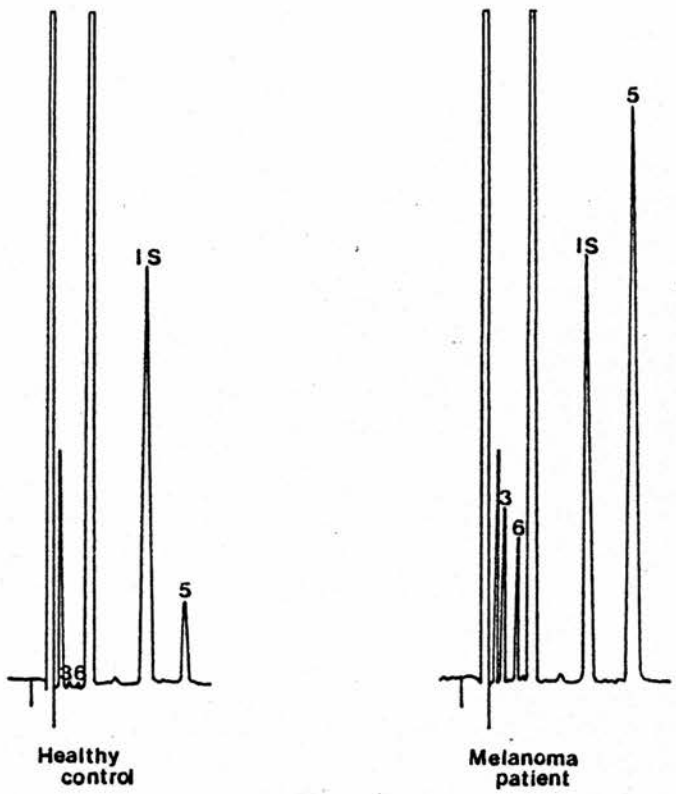

FIGURE 2.15 HPLC chromatograms of plasma extracts from a healthy individual and a patient with stage III melanoma showing the increase in size of a number of plasma peaks in the melanoma patient

IS = internal standard

5 = 5-S-CD

3 and 6 = other cysteinyldopas

and extracted as described on page 57. The extracts were analysed by HPLC and the results used to construct calibration curves. (Table 2.4).

Curves obtained for 5-S-CD in water and 5-S-CD in plasma were very similar indicating that extraction of 5-S-CD from these two media does not differ greatly and that matrix effects are insignificant. The use of water standards in day to day calibration of the assay was considered to be acceptable.

A set of five water standards was, therefore, prepared at concentrations ranging from 1·25 to 125 ng/ml, and 2ml portions of these, containing 5mM dithiothreitol, were stored at -20°C until required. A calibration curve was constructed using these five standards, which were extracted with each batch of samples, on every occasion that an assay was performed.

(b) Intra- and inter-assay precision and quality control

(i) Intra-assay precision

The intra-assay precision was established by extracting and analysing 10 portions of each of 2 plasma pools, one containing a low and one a high concentration of 5-S-CD. The results are given in Table 2.5.

(ii) Inter-assay precision

The inter-assay precision was established by accumulating quality control data, based on the analysis of one sample each of the high and low concentration pools in 23 consecutive analytical runs. The coefficients of variation for these groups of results are given in Table 2.6.

Table 2.4 Construction of calibration curves

Water standards

L-5-S-CD added (ng)	Ratio of peak height of L-5-S-CD to peak height of internal standard
2·5	0·26
25	1·64
62·5	3·61
100	6·02
125	7·38
200	11·76
250	14·95

Plasma standards

L-5-S-CD added (ng)	Ratio of peak height of L-5-S-CD to peak height of internal standard
2·5	0·32
25	1·49
62·5	3·70
100	5·85
125	7·28
200	11·94
250	15·08

Table 2.5 Intra-assay precision

	5-S-CD concn (nmol/l) Plasma 1	Plasma 2
Sample no. 1	11·7	200·3
2	11·0	223·0
3	11·7	212·5
4	11·0	216·9
5	10·5	206·4
6	11·2	207·6
7	11·7	212·0
8	10·5	202·5
9	11·2	230·5
10	11·4	220·5
Mean 5-S-CD (nmol/l)	11·19	213·22
Standard deviation	0·45	9·56
Coefficient of variation	4·05%	4·48%

Table 2.6 Inter-assay precision

	QC1	QC2
Mean 5-S-CD (nmol/l)	11·7	233·7
Standard deviation	0·44	9·2
Coefficient of variation	3·76%	3·94%

(iii) Quality control of the routine assay

Two plasma pools were prepared, one being at a high level of 5-S-CD, and one at a level which may be expected in healthy controls, both containing 5mM dithiothreitol. 2·5ml portions of each pool were prepared and stored at -20°C until required. Each time an assay was performed, a portion of each of the quality control pools was extracted and analysed with the other samples and standards. Results were recorded with previous quality control results.

Patients and controls

(a) Healthy volunteers

(i) Study of plasma 5-S-CD levels over a 12 hour period

The subjects involved in this study were 4 healthy volunteers, 3 males and 1 female, aged between 23 and 47 years. These subjects had 10ml blood samples taken at hourly intervals over a period of 12h, between 9am and 9pm. The subjects had fasted from the previous evening, but were allowed to eat normally after the first sample had been taken.

(ii) Study of plasma 5-S-CD levels over a 12 month period

The subjects described in this study were 11 healthy volunteers, 7 males and 4 females, aged between 23 and 49 years. These volunteers had 10ml blood samples taken at monthly intervals over a period of 12 months, between January and December 1983. The subjects were all resident in Edinburgh (latitude 56°N), although several of them had holidays abroad during the period of study.

(iii) Study of plasma 5-S-CD levels in individuals with different skin and hair colours

The white-skinned subjects with blonde or brown hair involved in this study were 9 of the healthy volunteers involved in the study of seasonal variation in plasma 5-S-CD. The 8 white-skinned subjects with red hair were also healthy individuals from the Edinburgh area. The 11 black-skinned, black-haired subjects of African descent were resident either in Edinburgh or Birmingham (latitude 53°N). These subjects all had a single 10ml blood sample taken.

(b) Patients with oculocutaneous albinism

20 patients with oculocutaneous albinism (OCA), 10 being

tyrosinase-negative and 10 tyrosinase-positive, had 10ml blood samples taken for estimation of plasma 5-S-CD. Of these 20 patients, the tyrosinase status of 3 was determined using a hairbulb tyrosinase test (Kugelman and Van Scott, 1961). The status of the remaining 17 patients was assessed by clinical means alone, tyrosinase-positive albinos being those with coloured irides, minimal hair pigmentation and scattered pigmented naevi, and tyrosinase-negative albinos being those with translucent irides, white hair, and no pigmented naevi.

(c) Patients with psoriasis undergoing treatment with UVR

The subjects studied in this investigation were patients undergoing UV treatment for widespread psoriasis. The patients were receiving either short-wave UVR (UVB: 280-315 nm) or photochemotherapy in the form of combined psoralen and long-wave UVR (UVA: 320-400 nm), commonly called 'PUVA' (Parrish, 1981). Psoralens are naturally-occurring, tricyclic, furocoumarin-like compounds, some of which can be activated by UVA to elicit phototoxic reactions. The rationale for the use of PUVA in the treatment of psoriasis is inhibition of the increased DNA synthesis within keratinocytes of the psoriatic lesions. This inhibition is accomplished by a specific interaction between psoralen and DNA molecules that involves absorption of radiant energy in the UVA range. Absorption of photons in the UVA spectrum transforms psoralen molecules into a photoexcited state, resulting in photoconjugation of psoralen with the pyrimidines of DNA and inhibition of DNA synthesis and, therefore, cell division (Parrish et al., 1978).

Treatment of individual patients was largely dependent on skin type, defined in terms of the response to exposure to natural sunlight as follows:- I - always burn, never tan; II - sometimes burn, eventually tan; III - rarely burn, usually tan; IV - tan, never burn.

Patients received treatment twice weekly at first, and less frequently thereafter, although there was some variation in frequency of treatment from one patient to another.

Patients receiving PUVA therapy were given 1·5 - 2·5 mg/kg body weight of 8-methoxypsoralen (8-MOP), which is a derivative of psoralen itself, and is the most commonly used in medicine (Parrish, 1981). The administration of 8-MOP was followed, after 2h, by irradiation using a Waldmann 6001 unit containing fluorescent tubes with an emission spectrum between 320 and 390 nm and a peak emission of 365 nm. Patients were started with a dose of 1 to 2 J/cm^2, depending on skin type and response to a test dose, and this was increased by 0·5 to 1 J/cm^2 per session to a maximum of 9·5 J/cm^2. Blood samples for 5-S-CD assay were taken before therapy was started, and thereafter at approximately weekly intervals.

The UVB light source was a Theraktin UV bath, with an emission spectrum of 270 to 310 nm, and a maximum of 290 nm. The intensity of radiation was estimated to be 0·45 mW/cm. The initial dose was approximately 0·15 J/cm, and this was increased by 0·05 to 0·2 J/cm per session, to a maximum of 1·35 J/cm, again depending on skin type. Blood samples for 5-S-CD assay were taken before therapy was started, and thereafter at approximately weekly

intervals.

The degree of pigmentation and erythema induced by the treatment, either absent, minimal or marked, was recorded for each patient, and the times at which these responses were seen were also recorded.

(d) Patients with psoriasis undergoing treatment with dithranol

The subjects involved in this study were 6 patients undergoing treatment with topical dithranol (1,8 - dihydroxy - 9 - anthrone). The mechanism of therapeutic action of dithranol in psoriasis is thought to be due to a demonstrable interaction with DNA (Swanbeck and Zetterberg, 1971). Dithranol often has an irritant action on the skin resulting in erythema, and it is this property which was of interest in this study. Oxidation of dithranol results in the formation of products which cause discolouration of the skin which is not related to tanning (Ponec and Hulsebosch, 1974).

The patients in this study were started on 0·1% dithranol, the concentration being increased gradually to 0·5 or 1%, depending on the degree of erythema produced and improvement of the psoriasis. Patients generally received treatment daily. The presence of erythema and its severity (minimal or marked) were noted. Patients had blood samples taken before treatment was started, and thereafter at approximately weekly intervals.

(e) Patients with malignant melanoma

The clinical staging of disease in the melanoma patients involved in these studies is as follows:-

Stage I - primary tumour alone

Stage II - local lymph node involvement ± primary tumour

Stage III - metastatic spread beyond local lymph nodes

(i) Patients with primary cutaneous malignant melanoma

22 patients with histologically proven clinical stage I primary cutaneous MM were involved in this study. There were 11 female and 11 male patients with ages ranging from 26 to 69 years. All had a single 10ml blood sample taken immediately before surgical removal of the tumour.

(ii) Patients undergoing follow-up after surgery for stage I or stage II disease

All patients seen at the melanoma follow-up clinic in the Dermatology Dept., Royal Infirmary of Edinburgh, between January and December, 1983, had a 10ml blood sample taken, for 5-S-CD estimation, at each attendance.

These patients can be divided into a majority who were being followed up after surgery for stage I MM (usually wide excision and grafting), and a few who were being followed up after surgery for stage II MM (usually radical block dissection of local nodes). All of these patients were clinically disease free after their respective surgical procedures, and attendance at the follow-up clinic was for the purpose of assessment of their general state of health, and investigation of possible signs of development of

metastatic disease.

There were 88 patients in this group, 82 with stage I and 6 with stage II MM. 25 of the patients were male, and 63 female, with ages ranging from 18 to 91 years, at the beginning of the study.

Any patient who was found to have what was considered to be a raised plasma 5-S-CD level (greater than 20nmol/l), was recalled to the clinic as soon as possible for a repeat blood sampling. A repeatedly high plasma 5-S-CD level was investigated further, with the patient being subjected to a chest X-ray and thorough clinical examination. Where indicated, a CAT scan (computerised axial tomography) was also performed to assess intra-abdominal lymph node enlargement.

(iii) Patients with clinical stage III MM

There were 26 patients in this group, all with known stage III MM at the time of study. 10 of these patients had skin or subcutaneous metastases often with involvement of local lymph nodes, and the remaining 16 had visceral metastases, with involvement of, for instance, brain, lung or liver. There were 13 males and 13 females in this study, with ages ranging from 26 to 77 years.

These patients had either a single 10ml blood sample taken, or sequential samples over a number of months, for estimation of plasma 5-S-CD levels. Note was made of any treatment which the patients were receiving at the time of sampling, and whether or not the patients were responding to treatment. Results of liver function tests (LFTs) were also recorded where obtainable. These included bilirubin, alkaline phosphatase and lactate dehydrogenase.

In a few of the patients, samples of secondary tumour were available for pigment assessment and this was carried out in the Dept. of Pathology, University of Edinburgh. Pigmentation was graded on a scale of 0-4, 0 being when there was no visible pigment on either haematoxylin or eosin staining or special staining for melanin by Masson-Fontana, and 1-4 being a subjective assessment of level of pigmentation. All assessments were performed by Dr. K. McLaren.

(f) Patients with non-melanoma tumours

38 patients with histologically proven non-melanoma malignant tumours, including breast, lung, stomach, and ovarian cancers, and lymphoma, had single 10ml blood samples taken for plasma 5-S-CD estimation.

10 patients with non-melanoma tumours and known metastatic involvement of the liver had single 10ml blood samples taken for estimation of plasma 5-S-CD. Where possible, results of standard LFTs were also recorded.

(g) Patients with abnormal liver function

14 patients with inflammatory, rather than metastatic carcinomatous, liver disease had 10ml blood samples taken for 5-S-CD estimation. 1 patient had 3 samples taken at 1 month intervals. Standard LFTs were also performed at the time of plasma 5-S-CD estimation.

(h) Patients with chronic renal failure

14 patients with chronic renal failure had blood samples taken

for 5-S-CD estimation. Plasma urea and creatinine levels were also measured at the time of estimation of plasma 5-S-CD.

(i) Patients with Parkinson's disease

One patient attending the melanoma follow-up clinic, following removal of a stage I melanoma, who was apparently disease free, was found to have a markedly raised plasma 5-S-CD level on 2 consecutive occasions. This patient also had Parkinson's disease and was, at that time, undergoing treatment with combined L-dopa and carbidopa ("Sinemet": Merck, Sharp & Dohme, Herts., UK). It was, therefore, decided to investigate other patients with Parkinson's disease to see if a raised plasma 5-S-CD was a common finding in these patients.

8 further patients with Parkinson's disease had 10ml blood samples taken for estimation of plasma 5-S-CD levels. 5 of these patients were undergoing treatment with "Sinemet", and the remaining 3 were being treated with amantadine ("Symmetrel": Geigy Pharmaceuticals, Sussex, UK). It was not possible to obtain samples from untreated Parkinson's patients.

RESULTS

Plasma 5-S-CD levels over a 12 hour period

The results of this study are presented in Fig.3.1. There was some variation in the mean plasma 5-S-CD concentration during the 12h period, the lowest mean value being 4·6 nmol/l at 1pm, and the maximum value of 6·3 nmol/l occurring at 4pm and 9pm. The slight increases observed in plasma 5-S-CD mid afternoon and evening may have been related to ingestion of meals. Comparison of the lowest and highest values observed throughout the day by means of an independent t-test showed these two sets of values to be statistically significantly different ($p < 0{\cdot}05$).

Plasma 5-S-CD levels over a 12 month period

The results of this study are presented in Fig.3.2. Plasma 5-S-CD concentrations were low during the winter, but began to rise during the spring months to reach a maximum mean value of 11·9 nmol/l in June. Thereafter the mean concentration fell to a minimum value in December.

The considerable variation between results from individual subjects at any time is reflected in the large standard errors. Comparison of the results for June and December, the highest and lowest mean values respectively, by means of an independent t-test, shows a statistically significant difference between these two sets of figures ($p < 0{\cdot}05$).

Plasma 5-S-CD concentrations in the 11 healthy individuals all of whom were resident in Edinburgh did not rise above 20 nmol/l, even during the summer months. This figure was taken to represent the upper limit of normal plasma 5-S-CD concentrations which could

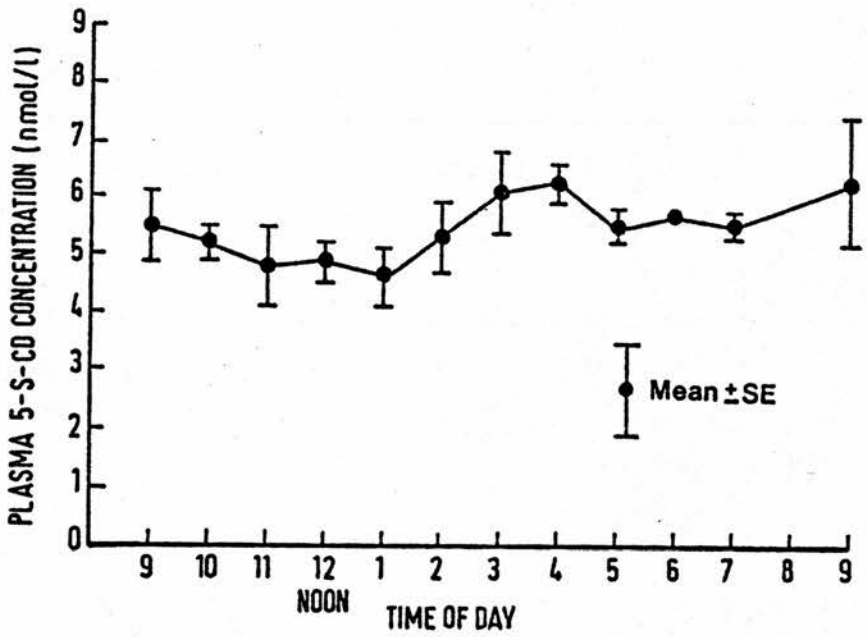

FIGURE 3.1 Plasma 5-S-CD levels in 4 healthy individuals over a 12h period

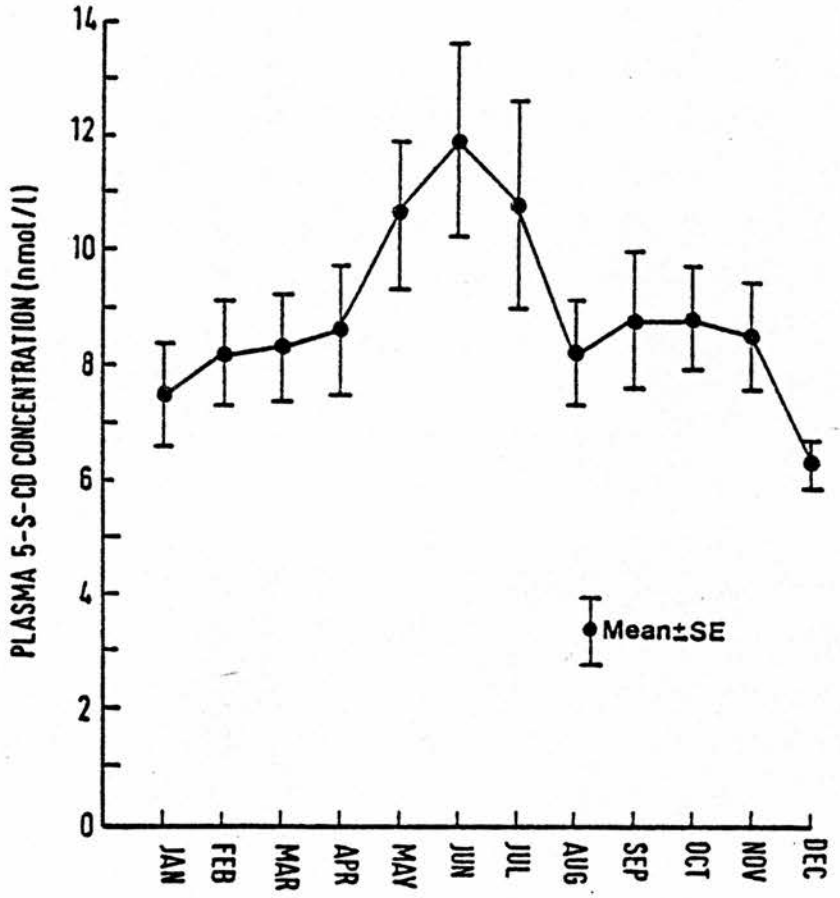

FIGURE 3.2 Plasma 5-S-CD levels in 11 healthy individuals over a 12 month period

be expected in patients being seen in clinics in and around Edinburgh.

There did not appear to be a significant rise in plasma 5-S-CD levels from pre- to post-holiday measurements in those of the subjects who had holidays abroad, and any change which was seen was comparable to that seen in those subjects who stayed in Edinburgh during the summer.

Plasma 5-S-CD levels in individuals with different skin and hair pigmentation

The results of this study are presented in Fig.3.3. All subjects were found to have plasma 5-S-CD levels which fell within the previously determined reference range. Statistical analysis of the results shows that there was no significant difference between levels in white-skinned subjects with blonde or brown hair and levels in white-skinned subjects with red hair, or between levels in white-skinned and black-skinned subjects (independent t-test, $p > 0{\cdot}05$).

Plasma 5-S-CD levels in oculocutaneous albinism

The results of this study are presented in Fig.3.4. All 20 patients were found to have plasma 5-S-CD concentrations which were within the reference range. Statistical analysis of the results showed that there was no significant difference between the results from normally pigmented controls and albinos, or between results from tyrosinase-negative and tyrosinase-positive albinos (independent t-test, $p > 0{\cdot}05$).

The effect of exposure to UVR on plasma 5-S-CD levels in psoriatic patients

The results of this study are presented in Tables 3.1 and 3.2,

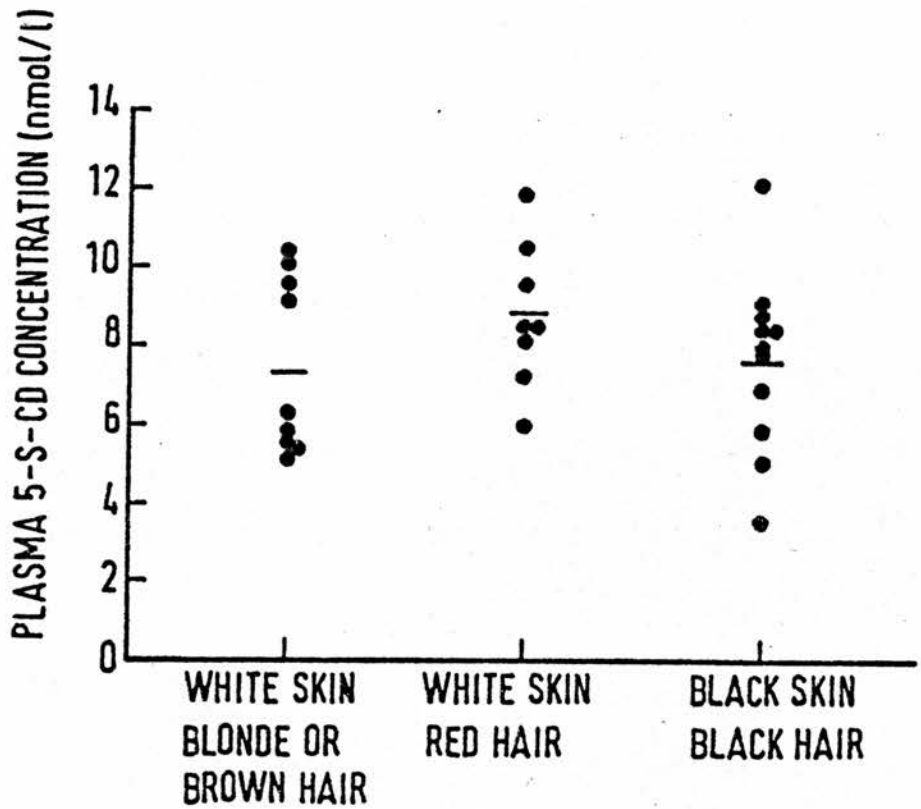

FIGURE 3.3 Plasma 5-S-CD levels in subjects with different skin and hair pigmentation (individual values plus bar representing mean value)

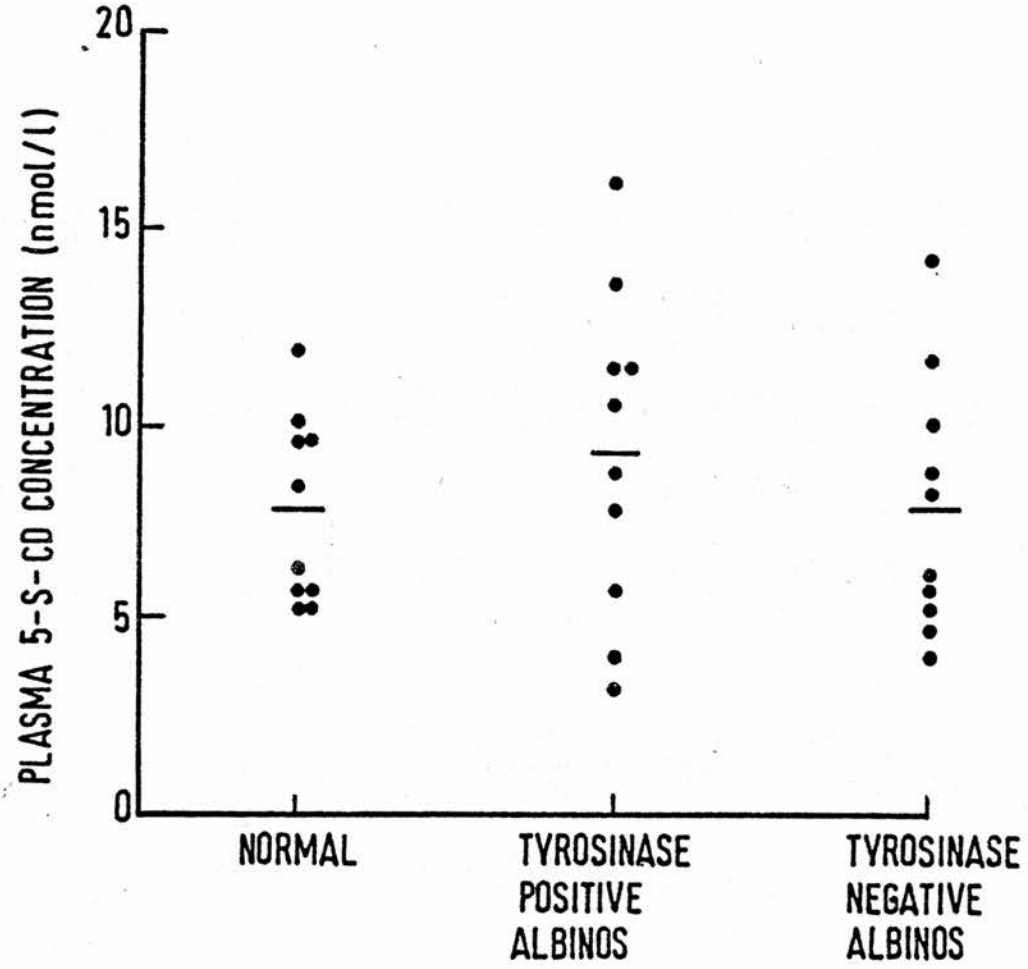

FIGURE 3.4 Plasma 5-S-CD levels in normally pigmented individuals and patients with tyrosinase-positive and tyrosinase-negative oculocutaneous albinism (individual values plus bar representing mean value)

Table 3.1. Sequential plasma 5-S-CD concentrations (nmol/l) in patients receiving UVB therapy

(percentage of pretreatment value shown in parenthesis)

Subject (skin type)	Total dose (J/cm^2)	Pretreatment	Number of treatments													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
W.B.(II)	7.1	7.8	17.4 (223)		15.1 (194)		16.1 (206)		8.8 (113)		5.8 (74)		5.5 (71)			
M.B.(IV)	4.5	10.0			17.3 (173)			10.5 (105)			7.2 (72)					9.7 (97)
R.K.(III)	1.9	11.2		16.8 (147)	27.1 (242)	13.7 (122)		11.6 (104)	11.0 (98)	7.9 (71)		9.7 (87)				
J.M.(III)	5.9	14.5		18.0 (124)	20.5 (141)		23.1 (159)	14.8 (102)		17.9 (123)	17.5 (121)		12.9 (89)	13.4 (92)		
A.R.(III)	6.2	15.2		21.8 (143)	37.7 (248)		28.2 (186)	28.1 (185)		22.8 (150)	23.0 (151)		21.8 (143)			
E.T.(IV)	6.5	8.0	14.1 (176)			12.8 (160)		17.3 (216)	4.6 (58)				7.9 (99)		7.9 (99)	
J.C.(ii)	3.4	12.2	28.6 (234)		27.8 (228)			18.9 (155)					10.2 (84)	19.3 (158)		

Table 3.2 Sequential plasma 5-S-CD concentrations (nmol/l) in patients receiving PUVA therapy

(percentage of pretreatment value shown in parenthesis)

Subject (skin type)	Total dose (J/cm^2)	Pretreatment	Number of treatments															
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
E.P.(IV)	159	7.6	14.5 (191)		36.9 (486)		29.9 (393)		16.4 (216		10.8 (142)	10.9 (143)	8.7 (114)	9.6 (126)	9.1 (120)	16.6 (218)	12.8 (168)	8.7 (114)
PMcA(II)	61.5	10.6	10.3 (97)		8.3 (78)			20.1 (190)	23.9 (225)	5.3 (50)		17.3 (163)		10.9 (103)				
I.I.(III)	104.5	9.5		18.0 (189)		20.0 (211)	14.7 (155)	12.5 (132)	12.0 (126)				14.8 (156)	9.1 (96)	13.5 (142)			
H.D.(II)	69.0	3.6	3.2 (89)		6.3 (175)		14.6 (406)		4.9 (135)	9.2 (255)	4.3 (119)		10.8 (300)		15.3 (425)		8.4 (233)	
J.R.(I)	58.5	6.3	17.1 (271)		16.7 (265)		17.6 (279)		13.8 (219)		15.8 (251)			17.6 (279)	14.8 (235)	11.6 (184)		11.6 (184)
W.B.(II)	119.5	11.3		14.6 (129)		11.6 (103)		14.0 (124)		21.4 (189)			13.2 (117)					
R.H.(IV)	71.3	10.7				11.7 (109)		15.1 (141)	20.4 (191)			14.8 (138)						

and in Fig. 3.5. All 6 of the patients treated with PUVA were found to have increased plasma 5-S-CD levels, occurring between 5 and 20 days after treatment was started, usually between the first and fifth treatments. Peak 5-S-CD levels, which were 2 to 5 times the pre-treatment levels, were reached, in most cases, between 10 and 20 days after the start of the treatment. Thereafter there was considerable individual variation in response to continuing treatment, with levels in some patients returning to approximately the pre-treatment value, whilst in others they continued to fluctuate throughout treatment.

UVB therapy also caused a rise in plasma 5-S-CD levels in all 6 patients studied. This rise tended to occur earlier than in the PUVA patients. The peak 5-S-CD values were between 1·5 and 2·5 times pre-treatment values, and the rises were overall considerably smaller than those seen in PUVA patients. The peak value tended to occur between 2 and 10 days after the start of treatment, and was generally followed by a return towards pre-treatment values shortly afterwards.

The response to treatment, in terms of the rise in plasma 5-S-CD levels, was generally observed, in both groups of patients, at a time when no tanning was as yet present, but there was often erythema.

Plasma 5-S-CD levels in psoriatic patients undergoing treatment with dithranol

The results of this study are presented in Table 3.3 and Fig. 3.6. Of the 6 patients studied, an increase in 5-S-CD levels was

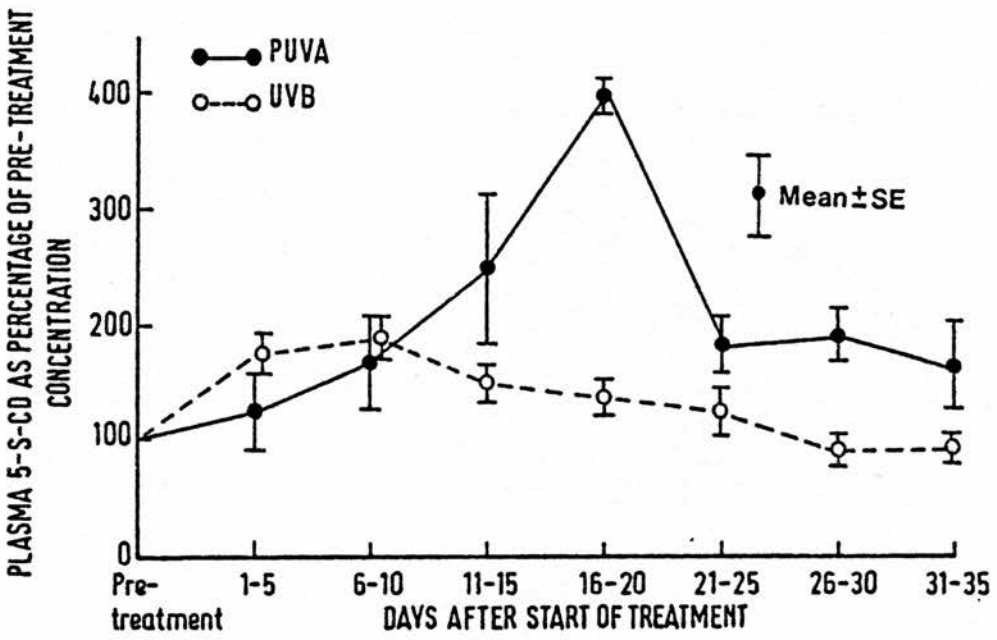

FIGURE 3.5 The effect of UVB and PUVA treatment on plasma 5-S-CD levels in psoriasis patients

Table 3.3 Plasma 5-S-CD levels in psoriasis patients undergoing treatment with dithranol

Patient	Pretreatment	Days after start of treatment															
		4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
HR	12.1			19.4 (160)			17.0 (140)										
MH	13.7				7.0 (51)							11.6 (85)					
BR	5.8		9.5 (164)							6.5 (112)							6.7 (116)
MR	8.8	7.2 (82)							8.1 (92)							8.4 (95)	
RM	5.5		5.6 (102)						4.7 (85)								
JC	6.0					12.7 (212)		13.6 (227)							7.5 (125)		

(Values in parenthesis percentage of pretreatment)

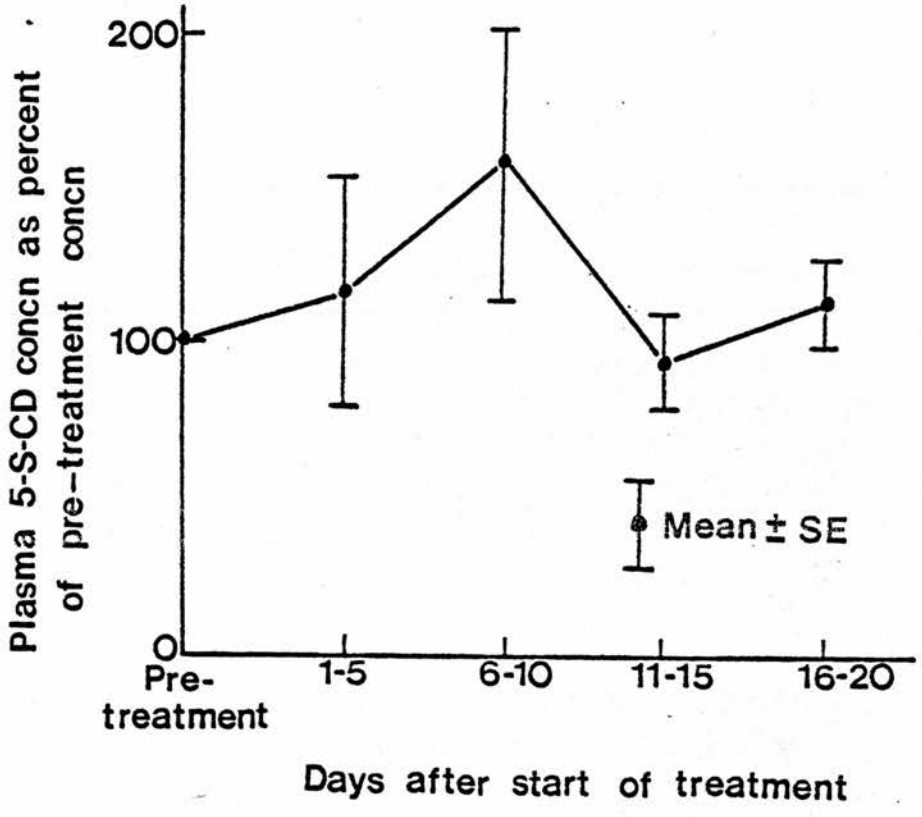

FIGURE 3.6 The effect of topical dithranol treatment on plasma 5-S-CD levels in psoriasis patients

seen in 3. The increase in mean 5-S-CD levels seen between days 6 and 10 after the start of treatment was not statistically significant when compared to the mean starting level (independent t-test, $p > 0{\cdot}05$). There did not appear to be any relationship between erythema and 5-S-CD levels.

Plasma 5-S-CD levels in patients with primary cutaneous malignant melanoma

The results of this study are presented in Fig.3.7. All 22 patients were found to have plasma 5-S-CD concentrations below 20 nmol/l which had been accepted as the upper limit of the reference range. The mean 5-S-CD concentration in these individuals was not significantly different from the mean in healthy controls (independent t-test, $p > 0{\cdot}05$).

Plasma 5-S-CD levels in clinically disease-free patients during follow-up after surgery for stage I and stage II MM

There were 88 follow-up patients in this study, and of these 4 developed confirmed metastases during the year of study; a further 5 developed metastases in the following 8 months. The results from these patients are presented in Table 3.4. Of the 4 patients who developed metastatic melanoma during the year of study, in 2 the first sign of progressive disease was a raised plasma 5-S-CD level (Fig. 3.8). In one of these patients (TI) this rise was observed 2 months before the patient began to feel generally unwell and the presence of liver and lung metastases was confirmed. In the second of these patients (HI) an increase in 5-S-CD was first seen 9 months before detection of metastatic disease, but this was followed by a return of the 5-S-CD level to within the reference range. The level did not increase again until 8 months later, 1 month before confirmation of the presence of liver and lung metastases. Both of these patients died within 1 month of clinical detection of secondary melanoma.

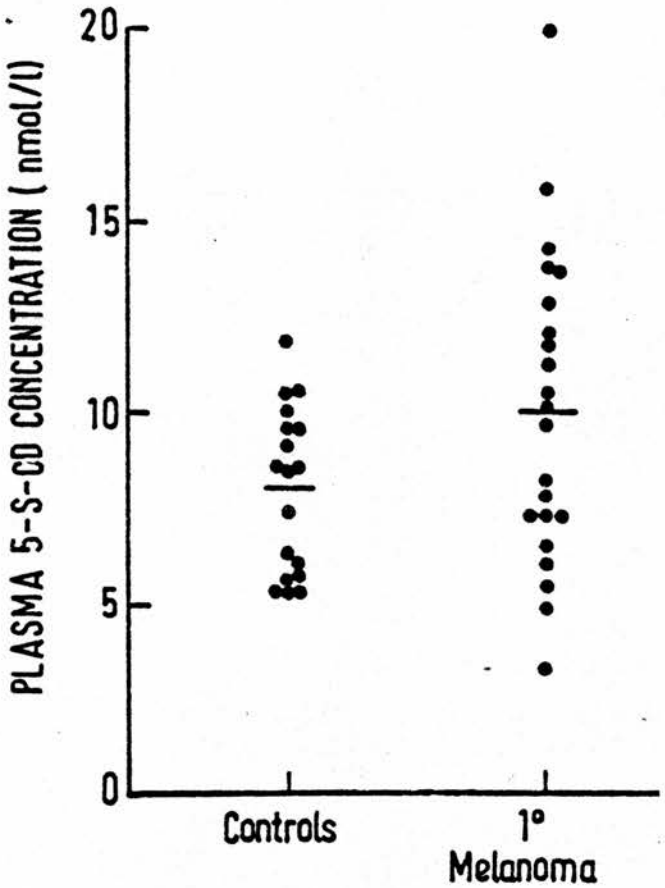

FIGURE 3.7 Plasma 5-S-CD levels in healthy controls and stage I melanoma patients (individual values plus bar representing mean value)

Table 3.4 Melanoma follow-up patients developing metastases between 1/83 and 8/84

Patient	Primary tumour		Secondary tumour		Plasma 5-S-CD levels	
	Date	Breslow (mm)	Date of confirmation	Site	Date	Concn (nmol/l)
MG	1979	2.7	9/83	Jejunum, colon, mesenteric lymph nodes		NR
HI	1982	4.1	12/83 - 1/84	Liver and lung	22/3/83 22/11/83	38.7 1181.7
TI	1979	3.0	12/83	Liver and lung	18/10/83	100.0
AS	1978	3.0	6/83	Liver		NR
GB	1976		6/84	L femur		NR
MB	1977	1.4	4/84	R foot (bone)		NR
DC	1983	8.5	8/84	Local skin		NR
EM	1980	< 0.5 (+ regression)	3/84	Spleen	17/1/84	25.7
SS	No demonstrable primary (presented with stage II disease)		8/84	L femur	29/11/83	24.6

NR = never raised

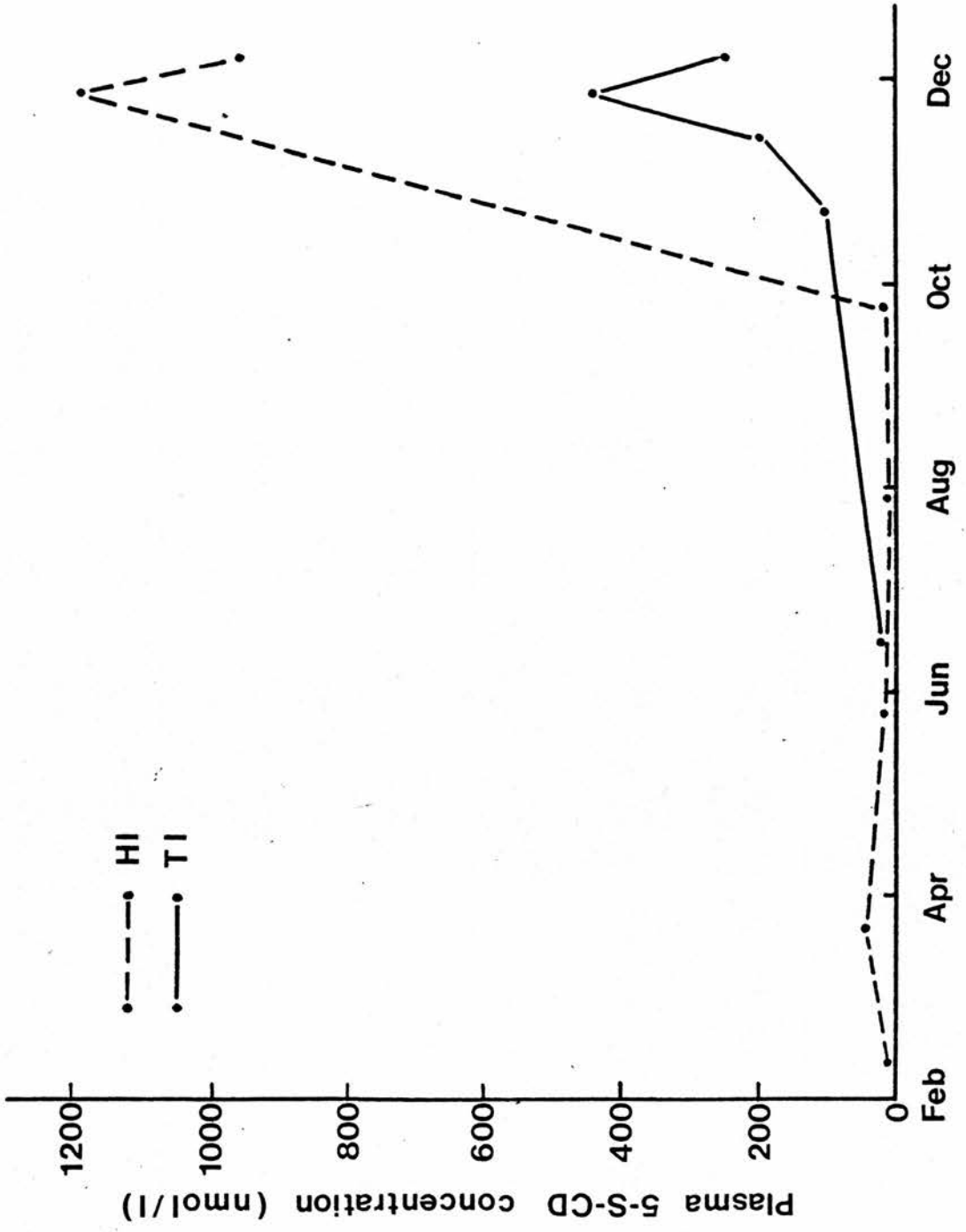
Plasma 5-S-CD concentration (nmol/l)
1200
1000
800
600
400
200
0
Feb
Apr
Jun
Aug
Oct
Dec
HI
TI

FIGURE 3.8 Plasma 5-S-CD levels in 2 patients (HI and TI) over a period of 11 months during which both patients developed stage III melanoma

The remaining 2 patients who developed metastases within the year of study had normal plasma 5-S-CD levels at each visit to the follow-up clinic. One of these patients (MG) had a large secondary tumour removed from her abdomen, which was barely pigmented macroscopically, and on histological examination was seen to have minimal evidence of melanin production. This was the only patient in whom metastatic tumour tissue was available for histological examination. The other patient in this category (AS) developed liver metastases.

Of the 5 patients who developed metastases after the conclusion of the 5-S-CD study, 2 had at some point been found to have abnormal 5-S-CD levels during the study. In one of these patients (SS) the increased level had been seen 9 months before metastases were eventually confirmed, and in the other (EM) the raised level had occurred 2 months before. The remaining 3 patients (MB, GB and DC) developed metastases 4, 6 and 8 months respectively after the completion of the study, but had not had raised 5-S-CD levels before the study ended.

Of the 79 follow-up patients who had not shown clinical signs of metastatic disease up until 20 months after the start of the study, 7 nonetheless had a raised plasma 5-S-CD level at some point during the study. Repeat sampling from these patients, however, resulted in the finding of normal plasma 5-S-CD concentrations, and no further investigations were performed.

Plasma 5-S-CD levels in patients with stage III MM

The results of this study are presented in Fig.3.9, with the values shown in the figure being the highest 5-S-CD level seen in each of the patients involved.

15 of these 26 patients were found to have raised 5-S-CD levels, some repeatedly, and 11 had levels which fell within the reference range, again sometimes on more than one occasion. All of the patients who were found to have raised plasma 5-S-CD levels at the time of the first blood sample had consistently raised levels, if subsequent samples were obtained. The same consistency was seen in patients who had normal 5-S-CD levels in the presence of metastatic disease.

Liver function test (LFT) results were noted where available in these patients. 10 of the 15 patients with elevated 5-S-CD levels also had abnormal LFTs, either lactate dehydrogenase (LDH) or alkaline phosphatase (ALP), or both (Table 3.5). The remaining 5 patients with raised 5-S-CD levels had normal LFTs. Of the 11 patients with normal 5-S-CD levels, 2 had abnormal ALP, 4 had normal LFTs, and for the remaining 5 there was no record of LFTs at the time of plasma 5-S-CD estimation. 67% of the patients with abnormal 5-S-CD levels, therefore, also had abnormal LFTs, whereas the proportion of patients with normal 5-S-CD levels and abnormal LFTs was lower, being 33% of the 6 patients in whom these results were available.

There was no evidence of impaired renal function in those patients for whom plasma urea or creatinine levels were available.

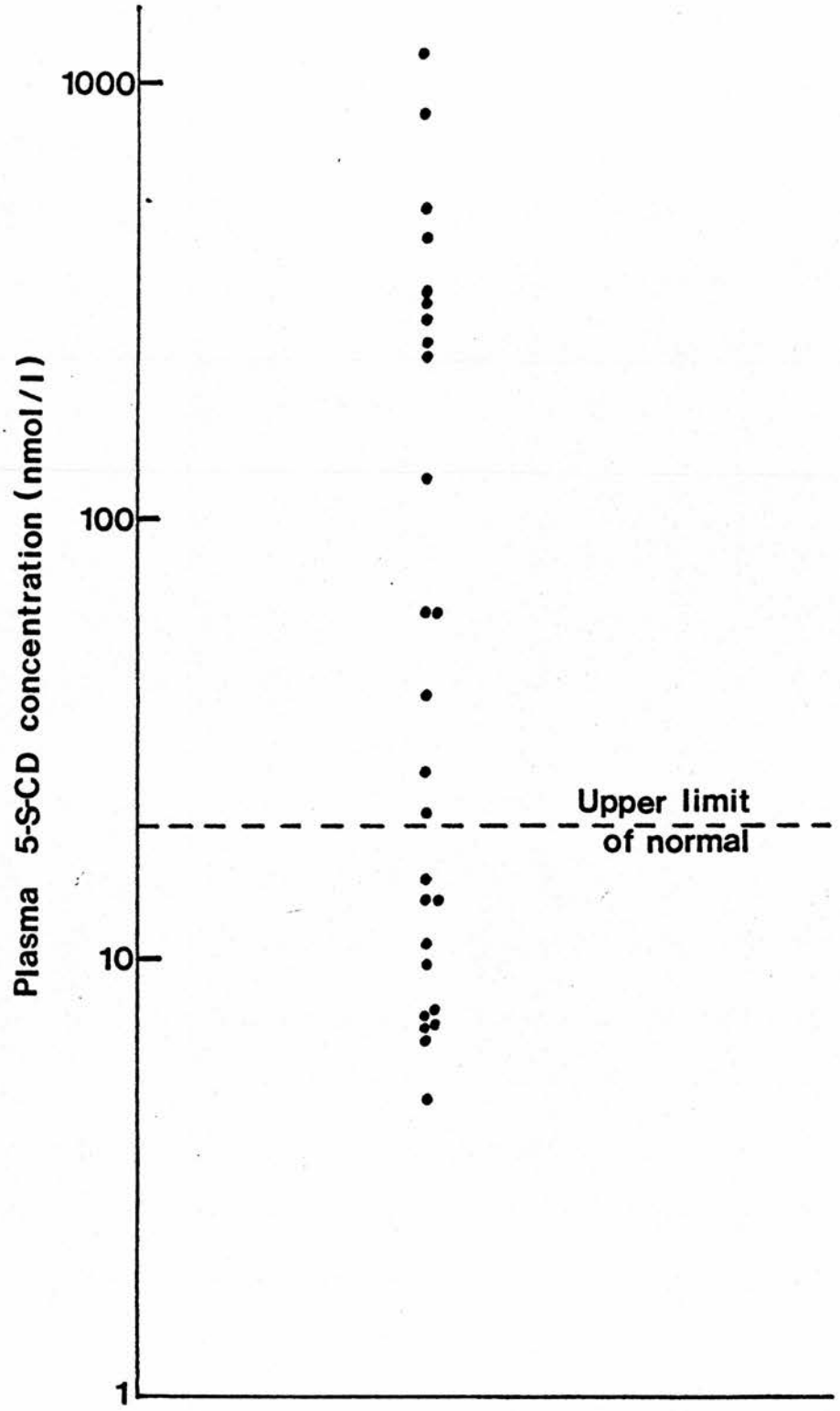

FIGURE 3.9 Plasma 5-S-CD levels in 26 patients with stage III melanoma (plotted on a log scale)

Table 3.5 Plasma 5-S-CD and LFTs in patients with stage III MM

Patient	Plasma 5-S-CD (nmol/l)	LDH (U/l)	ALP (U/l)
HI	950.4	815	334
AA	857.6	N	N
TC	522.2	857	191
MS	332.0	N	N
PS	315.0	N	N
HD	288.4	3680	163
MK	254.7	NA	154
TI	250.2	3585	900
GG	99.7	760	169
DB	83.1	475	N
CI	62.5	461	N
SF	60.9	N	N
MF	39.3	4310	N
EA	27.0	601	N
PM	21.6	N	N
MGr	15.0	NA	NA
RR	13.7	N	N
MB	13.6	N	N
RS	10.8	N	N
JG	9.5	N	194
DD	7.6	NA	NA
AD	7.0	NA	NA
HB	6.9	N	197
JM	6.6	NA	NA
MG	6.3	NA	NA
HG	4.8	N	N
Reference range		72-395	30-140

N = normal (within reference range)

NA = results not available

Plasma 5-S-CD levels in patients with non-melanoma tumours

The results of the study of non-melanoma tumours are presented in Fig.3.10. All 38 patients were found to have plasma 5-S-CD levels which fell within the reference range. The mean 5-S-CD concentration in each of the groups of patients was not significantly different from the mean in healthy controls, also shown in Fig.3.10 (independent t-test, $p > 0{\cdot}05$).

The results of the study of a separate group of patients with non-melanoma tumours and known metastatic involvement of the liver, are presented in Fig.3.11. All 10 patients were found to have plasma 5-S-CD levels which fell within the reference range. The mean concentration in this group of patients was not significantly different from the mean in healthy controls (independent t-test, $p > 0{\cdot}05$).

There was no relationship between LFTs and plasma 5-S-CD in these patients. Lack of relationship between plasma ALP (which was markedly elevated in a number of patients) and 5-S-CD indicates lack of relationship between intrahepatic biliary obstruction and raised plasma 5-S-CD.

Plasma 5-S-CD levels in patients with abnormal liver function

The results of this study are also presented in Fig.3.11. 3 of the 15 patients had raised plasma 5-S-CD levels, and the results from these patients are presented in Table 3.6 along with bilirubin, alanine amino transferase (ALT), ALP and creatinine in these patients. In 2 of these 3 the 5-S-CD was only marginally elevated; these patients had diagnoses of biliary cirrhosis and cirrhosis of unknown

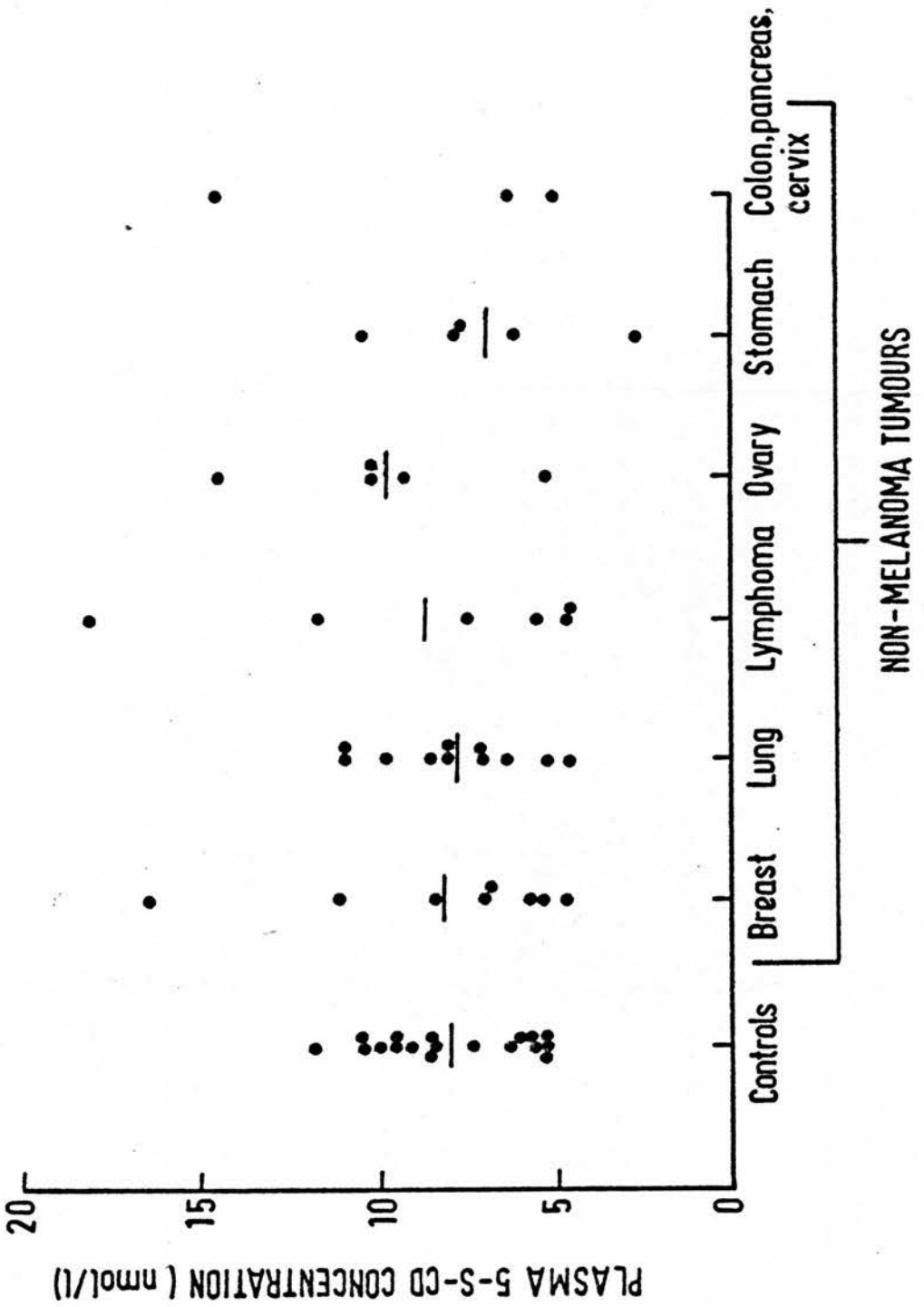

FIGURE 3.10 Plasma 5-S-CD levels in healthy controls and patients with non-melanoma tumours (individual values plus bar representing mean value)

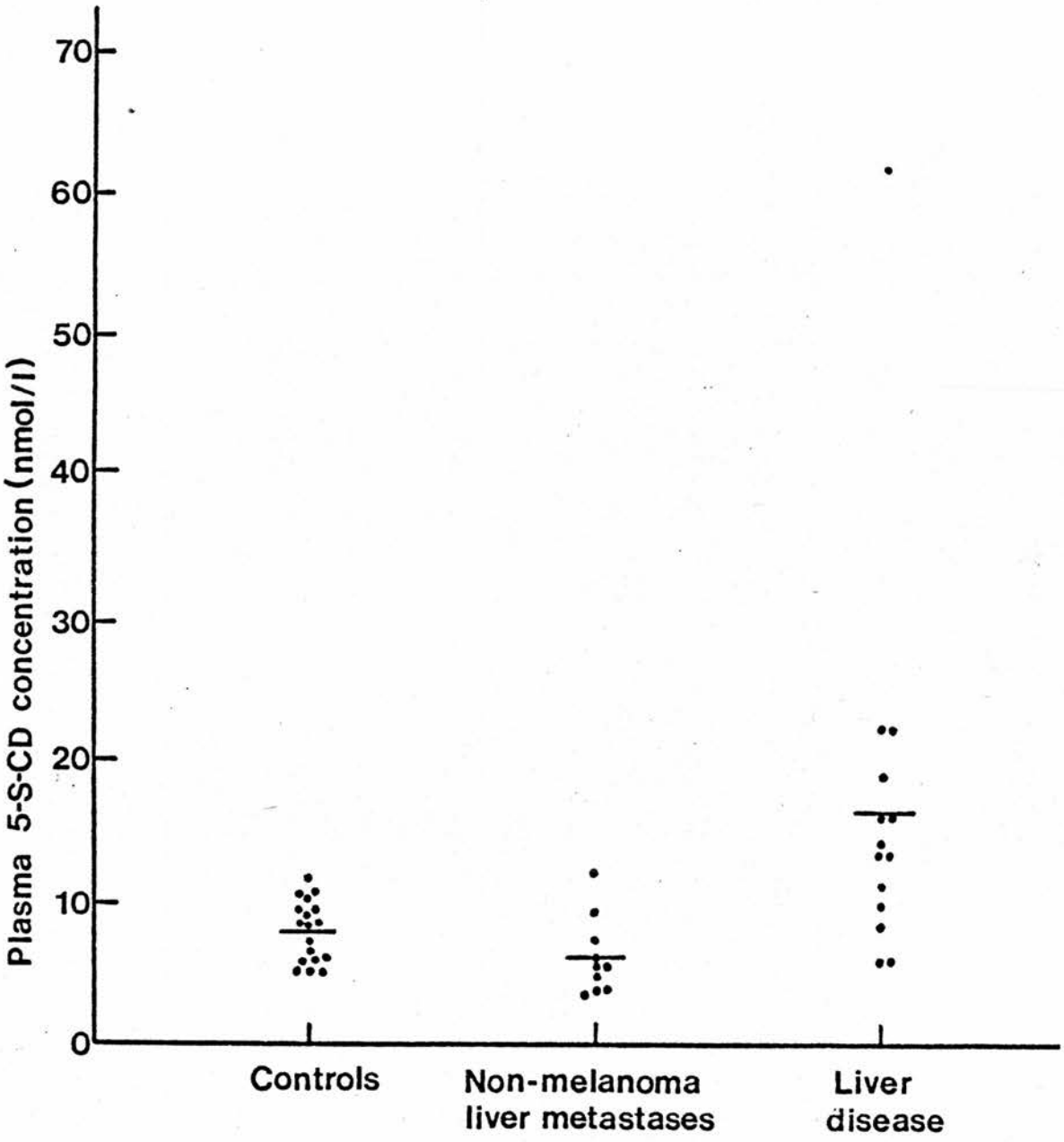

FIGURE 3.11 Plasma 5-S-CD levels in healthy controls and in patients with non-melanoma liver metastases and chronic inflammatory liver disease (individual values plus bar representing mean value)

Table 3.6 5-S-CD, LFT and creatinine levels in patients with liver disease

Patient	5-S-CD (nmol/l)	Bilirubin (μmol/l)	ALT (U/l)	ALP (U/l)	Creatinine (μmol/l)
MW	22.2	148	67	457	57
AW	22.3	27	35	89	60
JR (30/11)	62.3	64	91	350	72
JR (28/12)	20.2	62	72	308	89
JR (16/01)	9.6	53	88	338	93
Reference range		2-17	10-40	40-100	55-150

origin, respectively. In the third of these patients the plasma 5-S-CD was 3 times the upper limit of the reference range, at 62·3 nmol/l. This patients had alcoholic cirrhosis, with elevated bilirubin, ALT and ALP. A further 2 samples were obtained from this patient at intervals of 1 month, and the results are plotted in Fig. 3.12, showing a return of 5-S-CD levels to normal over this time whilst the LFTs remained elevated. There was no evidence of abnormal renal function in any of these 3 patients.

Of the 12 patients with normal 5-S-CD levels, 8 had raised ALP levels, indicating a lack of relationship between ALP, and therefore obstructive liver disease, and plasma 5-S-CD, in common with the findings in patients with metastatic carcinoma involving the liver.

Plasma 5-S-CD levels in patients with chronic renal failure

The results of this study are presented in Fig.3.13. 9 of the 14 patients were found to have raised 5-S-CD values. The plasma 5-S-CD concentrations and the plasma creatinine concentrations in these patients are shown in Fig.3.14. There was a significant correlation between these two variables (r= 0·878). LFTs in these patients were normal.

Plasma 5-S-CD levels in patients with Parkinson's disease

The results of this study are presented in Fig.3.15. All 6 patients being treated with L-dopa and carbidopa were found to have raised 5-S-CD levels, whereas the 3 patients being treated with amantadine had normal 5-S-CD levels.

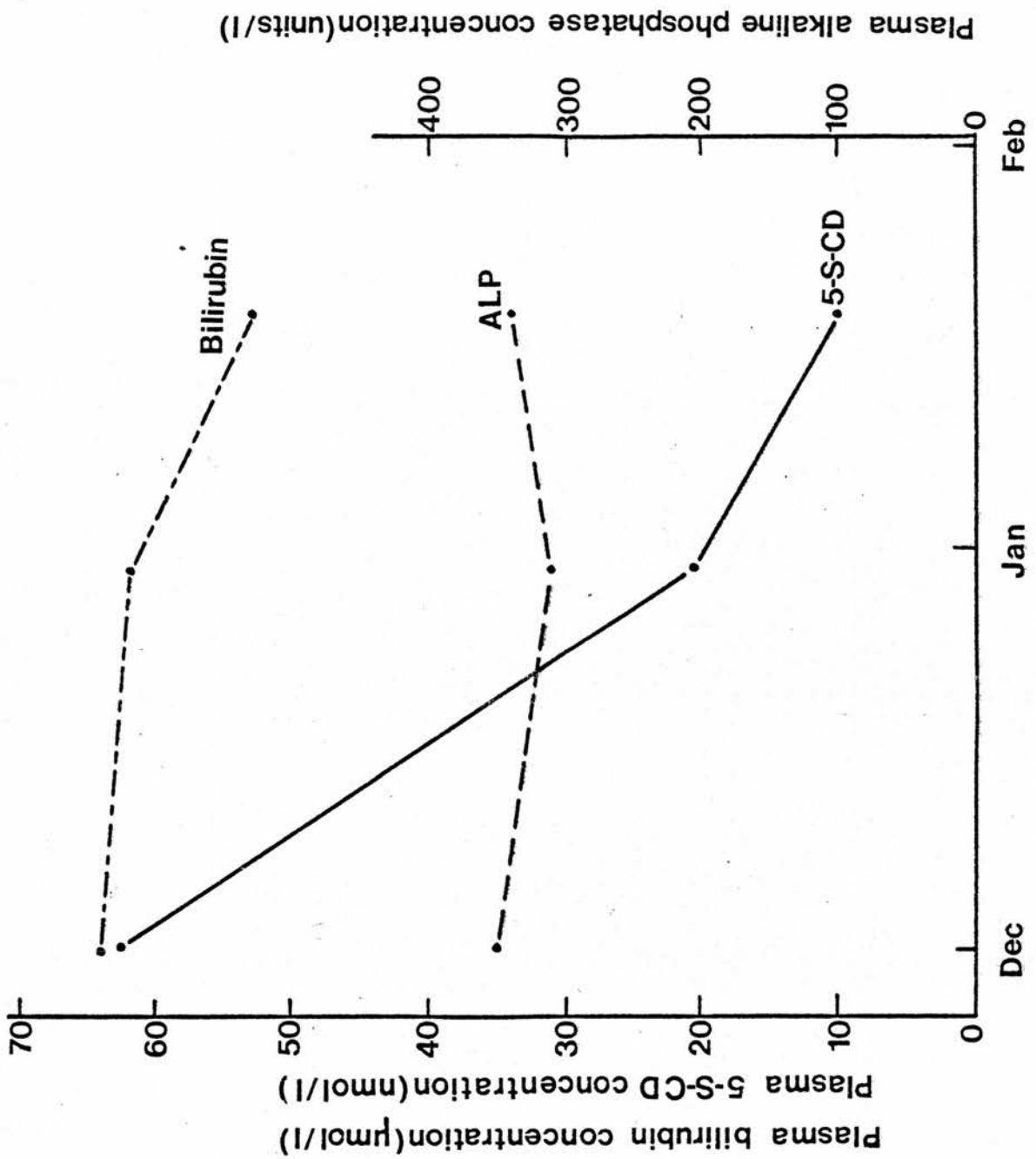

FIGURE 3.12 Plasma 5-S-CD, alkaline phosphatase and bilirubin concentrations in a patient with alcoholic cirrhosis of the liver, over a period of 2 months

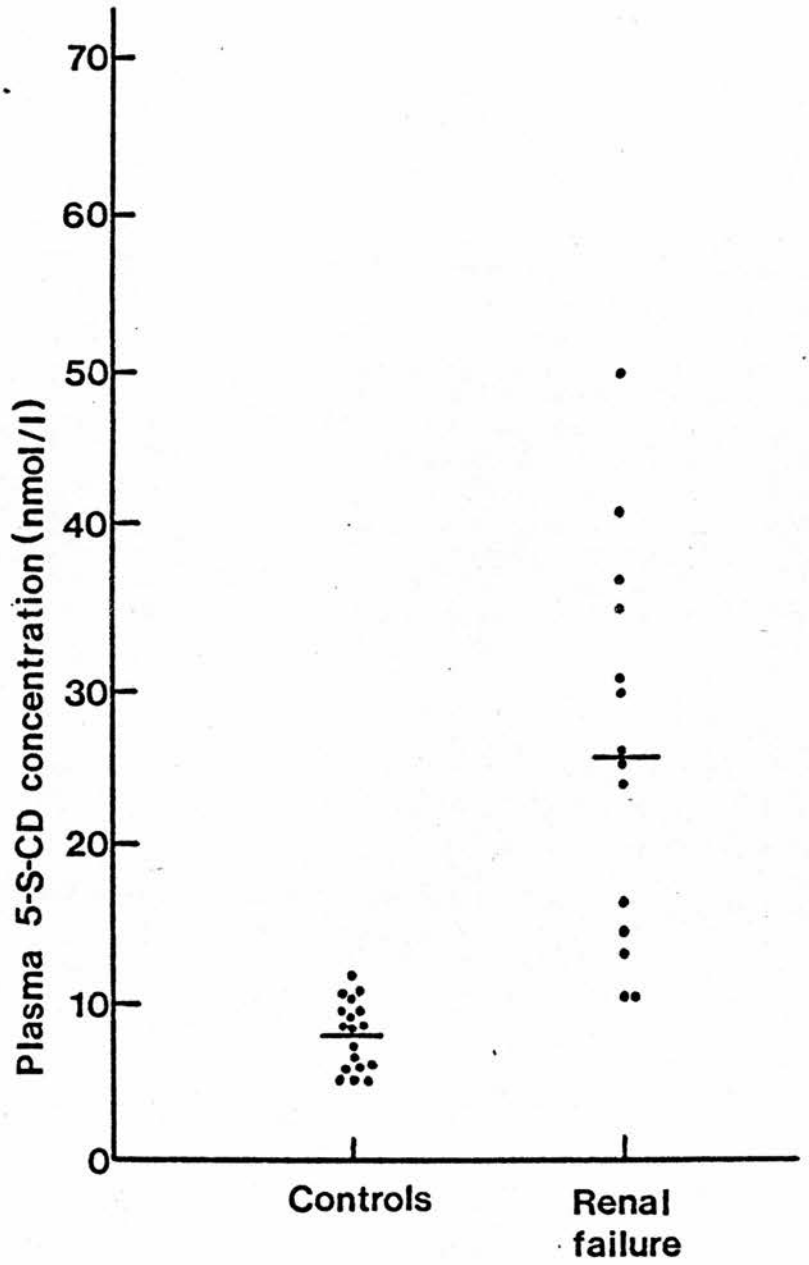

FIGURE 3.13 Plasma 5-S-CD levels in healthy controls and in patients with chronic renal failure (individual values plus bar representing mean value)

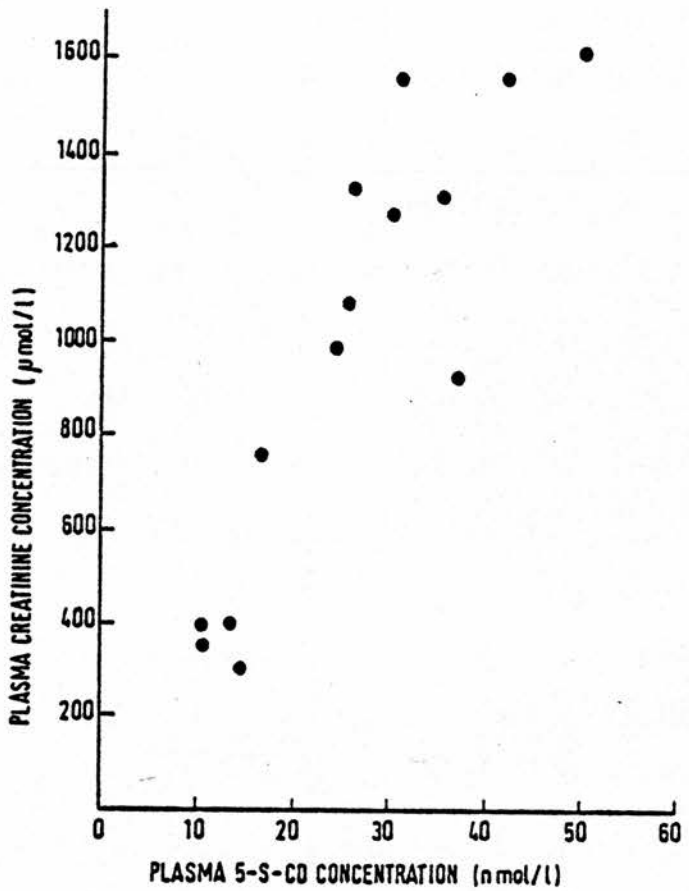

FIGURE 3.14 Relationship between plasma 5-S-CD and creatinine levels in patients with chronic renal failure (correlation coefficient, r = 0·88)

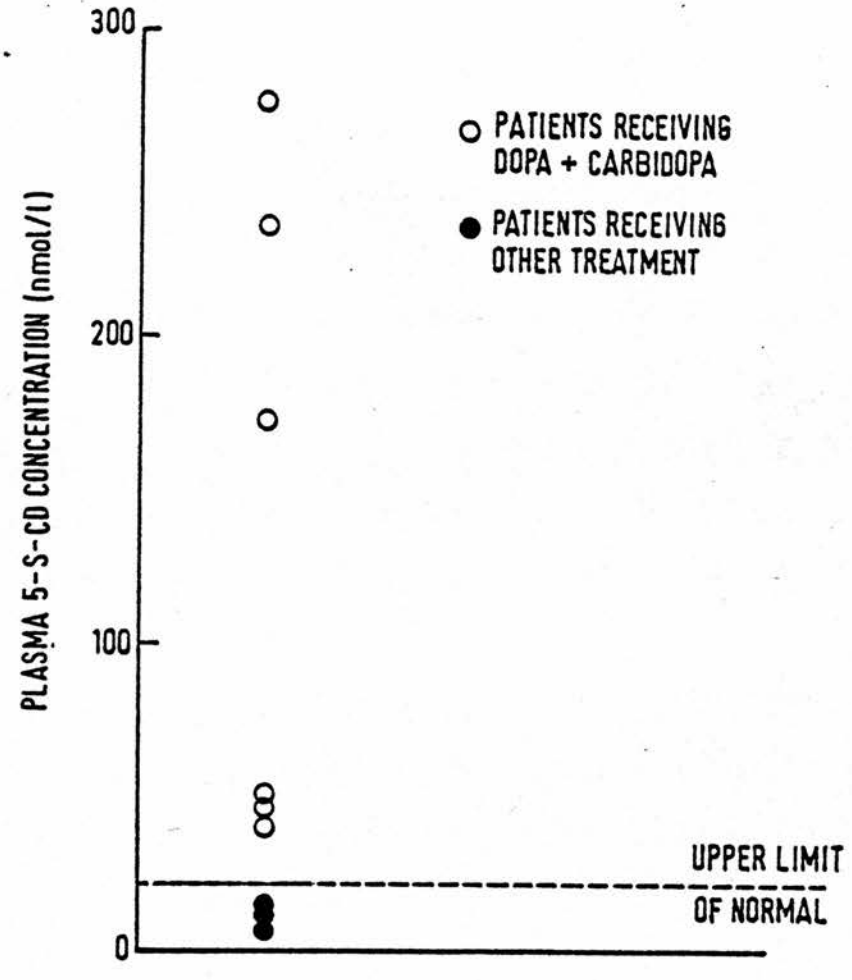

FIGURE 3.15 Plasma 5-S-CD levels in 9 patients with Parkinson's disease

DISCUSSION

METHODOLOGY

When the studies presented in this thesis were started, Agrup and coworkers, in Sweden, had already spent several years investigating urinary 5-S-CD levels in melanoma patients by means of a fluorimetric method (1979a). This method involved the adsorption of urinary catechols on to alumina, by the method of Anton and Sayre (1962), followed by oxidation of 5-S-CD using iodine to produce a fluorophore with maximum excitation at 325nm and maximum emission at 405nm. It was found that this method could distinguish 5-S-CD from other catechols, indoles and analogues of these compounds, and was, therefore, of high specificity for 5-S-CD. Fluorimetric assays performed in urine are, however, to some extent notorious because of the presence of many unknown fluorigens (derived from food or drugs) in urine. In the case of this particular fluorimetric assay, interference from certain drugs, such as salicylates, was found to occur (Hansson et al., 1978).

Prior to the studies presented in the Methods section of this thesis, fluorimetric assay of urinary 5-S-CD was examined briefly, using the method of Rorsman et al. (1973a). However, the only fluorimeter available at this time was found to give inconsistent results and to be insufficiently sensitive to detect the concentrations of 5-S-CD found in the urine of healthy individuals. In the absence of suitable equipment, therefore, the study of fluorimetric measurement of urinary 5-S-CD was not pursued further.

Banda et al. (1977) developed an assay for melanin-related metabolites, including 5-S-CD, in urine, using ion-exchange separation and colorimetric detection with the stable free radical diphenylpicrylhydrazyl (DPPH). An autoanalyser system, similar to that used by

Banda et al., was constructed. This system incorporated an ion-exchange column, a mixing coil for reaction with DPPH, a colorimeter with flow cell, and a chart recorder. Like the fluorimetric method, this method was found to be too insensitive to detect the concentrations of 5-S-CD found in the urine of healthy individuals. In addition to lack of sensitivity, the analysis of each sample took up to several hours, as all interfering substances had to be eluted from the column prior to addition of the next sample. This meant that only one or two assays could be performed each day, and this was not considered to be acceptable in the analysis of patient samples.

Preliminary reports of HPLC assay of 5-S-CD in serum and urine had appeared in the literature (Hansson et al., 1978, 1979a), and it seemed that this method was sufficiently sensitive to detect the concentrations of 5-S-CD found in the serum of healthy individuals, measuring as low as picogram quantities. HPLC apparatus was obtained and used, in the first instance, in the study of urinary 5-S-CD levels.

Extraction of urine, prior to HPLC, using the alumina extraction method of Anton and Sayre (1962), was found to result in very poor recoveries of 5-S-CD, often as low as 25 percent. As an alternative to this form of extraction, small columns containing ion-exchange resin were constructed and samples were put through these columns prior to assay. Recovery of 5-S-CD from these columns was found to be in the region of 60 percent. Isoprenaline was being used at this time as internal standard, due to its structural similarities to 5-S-CD, however its behaviour on these ion-exchange columns was not in parallel with that of 5-S-CD and recoveries of isoprenaline were much lower than those seen for 5-S-CD. It appeared, therefore, that isoprenaline, although a suitable internal standard as far as

the HPLC system was concerned, being well separated from 5-S-CD on the chromatogram, was not compatible with ion-exchange extraction of 5-S-CD from urine. Isoprenaline would only be a useful internal standard in the assay of 5-S-CD when the extraction method was one which specifically favoured catechol compounds, such as alumina extraction.

As an alternative to isoprenaline, the possibility of using radioactively labelled 5-S-CD as internal standard was considered. This was not available commercially and would therefore have to be synthesized. Investigation of the availability of labelled substrates demonstrated that synthesis of labelled 5-S-CD using commercially available ^{14}C-cysteine as substrate would result in a product with insufficient activity to allow detection of the very low mass required for use as internal standard.

A visit to the laboratories of Rorsman's group in Lund, Sweden, revealed that they had, in fact, begun to use 5-S-D-cysteinyl-L-dopa, (5-S-D-CD), a diastereomer of naturally-occuring 5-S-L-cysteinyl-L-dopa, as internal standard. This was felt to be a particularly suitable internal standard for use in the assay of 5-S-CD due to the close similarity between the two compounds. 5-S-CD is readily oxidised, and this may result in falsely low values in the measurement of 5-S-CD in biological samples. Oxidation may occur at a number of steps, including sample collection, handling, storage or assay. The similarity between 5-S-CD and its stereoisomer in terms of both physical and chemical properties means that oxidation of both isomers is likely to occur in parallel and that loss of 5-S-CD from a sample due to non-specific oxidation will be mirrored by a loss of internal standard, and will thus be compensated for in the

calculation of 5-S-CD levels.

5-S-D-CD was synthesized using the tyrosinase reaction described by Ito and Prota (1977). This reaction was found to be simple to perform, and ion-exchange chromatography of reaction products resulted in a solution containing approximately 98 percent pure 5-S-D-CD as determined by HPLC. This solution could be diluted and used as internal standard. 5-S-D-CD was found to be well separated from 5-S-CD in a standard solution under varying chromatographic conditions. However, in urine extracted using either ion-exchange or alumina, the interference from other metabolites, oxidised by the electrochemical detector under the same conditions as the 5-S-CD isomers, was found to be so great as to prevent adequate detection of both 5-S-CD and 5-S-D-CD in one urine sample. Under all of the HPLC conditions studied, interference from other urinary metabolites was a major problem, and a build-up of contaminants on the HPLC column resulted in these columns having a very short life. In the face of these problems in the assay of urinary 5-S-CD using HPLC, studies were begun to examine the assay of 5-S-CD in plasma or serum.

There are a number of advantages in the use of an assay involving plasma or serum over an assay involving the collection of 24 hour urine samples. Although the collection of urine samples is relatively simple from a patient in a hospital ward, the organisation of outpatients and study volunteers is not so easy. It is inconvenient to carry a bottle around for a whole day and difficult to remember to collect every specimen during that time. For this reason, 24 hour samples may be incomplete, resulting in inaccurate estimation of the substance under study. As far as the subject of

the study is concerned, therefore, the collection of a small blood sample is usually preferable to a 24 hour urine collection. From the point of view of analysis, a few millilitres of blood is more manageable than litres of urine. Measurement of 5-S-CD in a single blood sample does have disadvantages, however, when compared to its measurement in a 24 hour urine specimen, the most important being its ability to reflect only the state of 5-S-CD production and excretion at a single point in time rather than over a 24 hour period, and this should be borne in mind in interpretation of results.

Study of both serum and plasma suggested that there was no significant difference in 5-S-CD levels found in the two media, and it was decided that plasma was more convenient to use routinely. Modification of the alumina extraction procedure which had been used previously in the studies of urine, and use of 5-S-D-CD as internal standard, resulted in a procedure which could be used to give an extract of plasma which could then be simply analysed by HPLC. The HPLC system was modified from that of Hansson et al. (1979a) to give good separation of 5-S-CD from both interfering metabolites in plasma, and the internal standard. The alumina extraction procedure was such that a substantial number of samples could be extracted in one batch, ready for HPLC analysis. Extraction of both cysteinyldopa isomers from the plasma was found to be approximately 50 percent, which, although quite poor gave sufficient levels in the resulting extract to allow adequate detection and measurement. The similar extraction efficiencies for the two isomers demonstrated the suitability of 5-S-D-CD as internal standard in this procedure. The HPLC procedure was found to be reproducible, as demonstrated by the intra- and inter-assay coefficients of variation, both less than 5 percent. Quality control samples were analysed with each batch of

patient samples to check reproducibility and to allow for any bad set of assays to be disregarded.

The major problem with the assay was found to be in the day to day variation in performance of the electrochemical detector. This could sometimes be related to some extraneous interfering factor, but was often quite inexplicable. The main factors which seemed to affect the detector were draughts, and power surges caused by the switching on and off of other equipment in the laboratory. These factors were often difficult to control and made the assay difficult to perform. Often baseline noise was so great as to prevent reasonable determination of 5-S-CD levels, and although this could on some occasions be overcome by some simple procedure such as polishing the detector electrode or making up fresh mobile phase, on some occasions it was not possible to overcome these problems and the assay had to be postponed.

Other forms of detection linked to HPLC have been used in the measurement of catecholamines. Fluorimetric monitoring following post-column derivatisation has been used in the measurement of adrenaline, noradrenaline and dopamine (Honda et al., 1983). However, most of the fluorimetric methods examined have been found to be tedious to perform and somewhat subject to interference from dietary factors and drugs.

UV detection has also been used in the measurement of catecholamines but does not appear to be as sensitive a method as electrochemical detection (Mell and Gustafson, 1977).

The temperamental nature of electrochemical detectors when used at the high sensitivities required for the detection of 5-S-CD in plasma limits the usefulness of this method in routine analysis, particularly in situations where it may have a number of different

operators, none of whom can afford to spend a lot of time sorting out difficulties in the day to day running of the system. However, in the situation presented here, where the method was being used as a research tool, and where there was only one operator who was able to spend time sorting out problems, this method was found to be both sensitive, detecting low picogram quantities of 5-S-CD, and specific for detection of this compound.

PLASMA 5-S-CD STUDIES

Having developed a method for the detection and measurement of 5-S-CD in plasma, and determined its performance and some of the problems likely to occur in its use, the next step was to use the method to obtain some background information about plasma 5-S-CD.

Plasma 5-S-CD in healthy volunteers

Measurement of plasma 5-S-CD levels in healthy volunteers over a period of time led to the acceptance of 20 nmol/l as the upper limit of normal 5-S-CD levels.

The demonstration by Rorsman et al. (1976) and by Nixon (1978) of the sensitivity of urinary 5-S-CD levels to exposure of the skin to sunlight prompted the study of plasma 5-S-CD levels in the climate of S.E. Scotland. The study extended over a 12 month period in an attempt to determine whether plasma 5-S-CD levels varied with season, as shown with urinary 5-S-CD in other climates, particularly that of S. Sweden (Rorsman et al., 1976).

Measurement of 5-S-CD levels over a 12 hour period demonstrated minimal variation throughout the day, making it unnecessary to obtain samples at a particular time of day (this is important when using a single blood sample in place of a 24-hour urine sample).

The time of month at which plasma samples were taken for the seasonal study depended solely on when subjects were available, and samples from different volunteers were, therefore, obtained at different times of the month. A significant increase in plasma 5-S-CD levels was observed during the summer months, with a mean of 6·3 nmol/l in December rising to a mean of 11·9 nmol/l in June. The largest increase in 5-S-CD level seen in an individual subject

was just less than 3-fold.

The increases in urinary 5-S-CD concentrations observed by Rorsman et al. (1976) were more marked than the increases in plasma 5-S-CD seen here, with a mean of 60 µg/24h in winter rising to a mean of 191 µg/24h in summer. In some subjects, 4-5 fold increases in 5-S-CD excretion were observed.

In common with the Swedish study it was seen here that the scatter of 5-S-CD values was greatest during the summer months, although the subjects who had the highest values in winter tended to also have the highest values in the summer.

A number of the subjects who participated in the Edinburgh study travelled abroad for holidays during the summer months. Plasma 5-S-CD was measured before the holiday and immediately on return from holiday. Plasma 5-S-CD levels in these subjects on return from abroad were no higher, in general, than those seen in subjects who had not travelled abroad on holiday.

Nixon (1978) demonstrated that increases in urinary 5-S-CD levels as high as 10-fold may occur several days after initial exposure to sunlight, but that this level will return to baseline within a matter of weeks after irradiation. It may be that although increases in plasma 5-S-CD levels occurred in the subjects in the seasonal study who travelled abroad and became tanned, by the time these subjects returned home and had a post-holiday 5-S-CD measurement, the levels had already returned to baseline, or were decreasing towards baseline levels. This explanation would be consistent with later findings in patients with psoriasis undergoing treatment with

UVB (which is similar to the UVR that volunteers would have received on holiday), in whom the increase in plasma 5-S-CD levels seen on exposure to UVB occurred early, reaching a peak 2 to 10 days after the start of treatment, and thereafter returned towards pre-treatment levels (Fig.3.5). The results from these subjects were included in the study although the subjects had not been exposed solely to the climate of Edinburgh. It seemed, however, that the major factor influencing 5-S-CD levels in the subjects included in this study was the natural sunlight in Edinburgh, and this was sufficient to produce a significant increase in 5-S-CD levels during the summer months, in all subjects under study. Although significantly increased, the levels in these subjects did not exceed the arbitrarily chosen upper limit of normal values of 20 nmol/l. It seemed, therefore, that in choosing this upper limit of normal values, even during the summer months 5-S-CD levels would not be expected to rise above the limit. It must not be forgotten, however, that this chosen level is an arbitrary one and that levels exceeding it should still be interpreted with caution, particularly during the summer months.

Plasma 5-S-CD and UVR

The study of psoriasis patients undergoing treatment with UVB or PUVA demonstrated the marked effect that exposure to large amounts of UVR can have on plasma 5-S-CD levels.

The reaction to PUVA therapy varied considerably from one patient to another. In some patients the change in 5-S-CD levels was seen as a rapid increase, reaching a peak several days after the start of treatment, followed by a return to pre-treatment levels over the

next few days. However, in other patients this rise in 5-S-CD was followed not by a return to baseline but by continually fluctuating levels throughout the remainder of the course of treatment. The initial results of this study seemed to point to a relationship between the skin type of the patient and the changes in plasma 5-S-CD levels seen in response to PUVA therapy. Patients with skin types III or IV seemed to exhibit the first pattern of 5-S-CD change, with a rise in levels followed by a return to baseline. Patients with type I or II skin, however, seemed to have plasma 5-S-CD levels which fluctuated throughout treatment. The apparent relationship between skin type and plasma 5-S-CD could be explained by considering the response of these groups of patients to UVR in terms of tanning and erythema. In patients with skin types III and IV, initial exposure to UVR could stimulate the melanocytes to produce the increased 5-S-CD levels seen initially, but as melanogenesis itself resulted in increased pigmentation of the skin, with consequent absorbance and scattering of UVR, the UVR would be prevented from influencing the melanocytes to the same extent and 5-S-CD production would, with decreased stimulation, return to normal. Patients with skin types I and II, however, develop little or no skin pigmentation in response to UVR, so no barrier is produced to the UVR, and subsequent doses are able to stimulate production of 5-S-CD by the melanocytes in the same way as the initial dose, resulting in fluctuating plasma 5-S-CD levels throughout the course of treatment in these patients.

These findings, and the hypothesis formulated on the basis of

them, were not, however, supported by study of further patients, in whom the response to PUVA and its relationship with skin type did not appear to be clear cut.

The response of patients to UVB therapy was less marked than the response to PUVA therapy. Although the doses of UVB received by these patients were an order of magnitude smaller than the doses of UV received in the PUVA study, it is not possible to compare directly the two forms of therapy and attribute the smaller responses in UVB patients simply to the smaller doses of UVR received. UVA and UVB have been shown to cause distinct and specific cellular and subcellular changes in the epidermal melanin unit (Pathak et al., 1976). UVA has been shown to cause less erythema and to be more effective in induction of melanogenesis than UVB. Also, in PUVA therapy, UVA is given in conjunction with 8-methoxypsoralen, which is able to enhance the effects of UVA on melanogenesis.

The initial rise in 5-S-CD, in both groups of patients, occurred before any tanning was present, and it seemed, therefore, that the increased 5-S-CD was not related to the tanning induced by the UVR exposure. The rise in 5-S-CD occurred, however, at a time when erythema was often present, particularly in patients with skin types I and II. This may indicate some association between increased 5-S-CD levels and skin inflammation or damage, as suggested in a number of studies by other workers (Hansson et al., 1981; Tegner, 1983; Tegner et al., 1983a, b). Alternatively, the initial rise in 5-S-CD levels may have occurred as a result of stimulation of melanogenesis by UVR, with intermediates such as 5-S-CD being released

into the bloodstream before any increase in pigmentation had occurred.

The possible association between plasma 5-S-CD and skin damage suggested by Tegner (1983) is not supported by this study in that the patients with the most marked rises in plasma 5-S-CD in response to UVR were not necessarily those who developed the most marked erythema. However, to investigate further the possibility that cutaneous cell damage may bring about increased 5-S-CD levels in the plasma, 6 psoriasis patients receiving topical dithranol were studied. This treatment often induces an irritant erythema which, although limited to the areas treated, is similar to that induced by exposure to UVR. Although slight rises in plasma 5-S-CD levels were observed in 3 of these 6 patients, no significant relationship between appearance of dithranol-induced erythema and rises in plasma 5-S-CD levels could be demonstrated.

It seems likely that the changes in plasma 5-S-CD concentration which occur in response to UVR are not due to any single factor, but may be due to a combination of stimulation of melanogenesis and cutaneous cell damage, involving keratinocytes and melanocytes, both of which can occur as a result of exposure to UVR.

Pathak et al. (1976) have examined the effects of UVR on skin-pigmentation at the microscopic level. Light and electron microscopic observations on delayed tanning reactions, induced by single or multiple exposures to UVA, UVB or UVA plus 8-methoxypsoralen (PUVA), were examined in vivo in human skin, biopsied following exposure to UVR. A single exposure to any of these treatments did not cause an increase in the number of functional melanocytes but

caused an increase in the synthesis and melanisation of melanosomes. Multiple exposures caused a marked increase in number of melanocytes, synthesis of melanosomes and degree of melanisation. Increased tyrosinase activity following UVR was demonstrated by incubation of the skin specimens with dopa and observation of an increase in melanin pigmentation. A biproduct of this increased tyrosinase activity in melanocytes is likely to be 5-S-CD, and increased excretion of this intermediate may well be observed before increased skin pigmentation itself is apparent.

As far as skin damage is concerned, part of the mechanism of cell damage by UVR appears to involve lysosome membrane damage and release of the lysosomal contents into the cell cytoplasm (Daniels and Johnson, 1974). The possibility that melanosomes situated in the keratinocytes are also damaged by exposure of the skin to UVR seems likely, as demonstrated by the presence of melanin in the cytoplasm of so-called 'sunburn cells' (Daniels and Johnson, 1974). Whether melanin precursors normally present in melanosomes may, therefore, be released by the damaging effects of UVR, is not clear from these studies.

Another possible explanation for some of these findings lies in the known production of reactive free-radicals in the skin in response to UVR (Norins, 1962; Pathak and Stratton, 1968), and the possibility that these molecules may have a part to play in 5-S-CD production is discussed on page 135.

Plasma 5-S-CD in relation to skin and hair colour

Comparison of plasma 5-S-CD levels in individuals with red hair, with levels in individuals with blonde or brown hair, demonstrated

that there was no significant difference between the results from the 2 groups. The predominance of the red/yellow phaeomelanins in the hair of red-headed subjects suggested that these subjects may be producing larger amounts of phaeomelanin precursors, such as 5-S-CD, than subjects with other hair colours. Red hair has, however, been shown to contain no 5-S-CD that can be extracted with perchloric acid, although trichochromes B and C have been isolated from red hair (Rorsman et al., 1979). These results are similar to the findings of Agrup et al. (1975b) who demonstrated that there was no statistically significant difference in urinary excretion of 5-S-CD between subjects with red, blonde or dark hair This was further supported by findings of comparable urinary 5-S-CD levels in healthy Japanese subjects (Morishima and Hanawa, 1981).

The 5-S-CD concentrations seen in the plasma of black-skinned subjects were similar to those seen in white-skinned subjects. This finding is predictable since there is no disparity in the melanocyte numbers between blacks and whites, the pigmentary differences being mainly due to differences in number, size, distribution and breakdown of melanosomes (Billingham, 1949; Szabo et al., 1969). Excretion of 5-S-CD in the urine of dark-skinned subjects was noted by Agrup et al. (1979b), and in this case was found to be lower than excretion in white-skinned individuals.

It appears from this study of skin and hair colour that differences in these factors, as genetically determined, do not result in differences in plasma 5-S-CD levels.

Plasma 5-S-CD in oculocutaneous albinism

Any study of pigmentation would not be complete without including a group of individuals in whom melanin pigmentation is either greatly reduced or completely absent, such as individuals with oculocutaneous albinism. Study of 20 such patients, 10 with tyrosinase-negative (ty-neg) and 10 with tyrosinase-positive (ty-pos) OCA, produced some unexpected findings. Ty-pos albinos were found to have 5-S-CD values which did not significantly differ from the values seen in normally pigmented individuals. The defect in ty-pos OCA is unknown, and tyrosinase activity in the hairbulbs of these individuals is apparently unaffected (King and Witkop, 1976). Normal 5-S-CD levels in these individuals can, therefore, be easily explained. Normal 5-S-CD values in ty-neg albinos are, however, more remarkable, in light of the lack of active tyrosinase in these individuals (King and Witkop, 1976). Aquaron *et al.* (1981) have demonstrated normal urinary excretion of 5-S-CD in ty-neg albinos in the sunny climate of Cameroon, in keeping with these results.

Although ty-neg albinos do not have the enzyme tyrosinase in their melanocytes, they do have the enzyme tyrosine hydroxylase in sympathetic nerve endings and adrenals. This enzyme catalyses the conversion of tyrosine to dopa, and is necessary for the production of catecholamines. Ty-neg albinos are, therefore, able to produce dopa. In order for the production of 5-S-CD to proceed, this dopa must be oxidised to the reactive dopaquinone. Since the addition of cysteine or other sulphydryl compounds to o-quinones is a rapid spontaneous reaction, any oxidising system with the capacity to convert catechols to o-quinones would be able to cause conjugation

of catechols with sulphydryl compounds. Several such systems are known, and their ability to bring about cysteinyldopa formation has been examined (Ito and Fujita, 1981a, b, 1982). Ito and Fujita have examined a number of systems, including peroxidase, superoxide and hydroxyl radicals (O_2^- and $OH^\cdot$), and iron-EDTA complex. Peroxidase was shown to be capable of bringing about the formation of cysteinyldopas *in vitro* under the conditions studied. This enzyme is detectable in a variety of cells, and has even been implicated in mammalian melanogenesis (Okun *et al.*, 1973). However, there is much evidence to suggest that the enzyme in question in this situation is tyrosinase rather than peroxidase (Hearing and Ekel, 1975; Smith and Swan, 1976).

A superoxide radical (O_2^-) is generated in many biological processes, and acts as an oxidant on a number of compounds, including catechols and sulphydryl compounds. Ito and Fujita studied the superoxide radical generated by the action of xanthine oxidase on hypoxanthine, to see whether this radical was capable of mediating the formation of cysteinyldopas from dopa and cysteine. They found that the O_2^- radical generated in this way reacted with dopa to produce cysteinyldopas at a much faster rate than its reaction with cysteine to produce cystine.

The hydroxyl radical ($OH^\cdot$) generated from the iron-EDTA complex (Fe^{2+}-EDTA) and hydrogen peroxide (H_2O_2) was also found to be capable of bringing about conjugation of dopa with cysteine.

These results demonstrated that cysteinyldopa formation can be mediated *in vitro* by peroxidase, superoxide and hydroxyl radicals, and by oxygen in the presence of iron chelate (perhaps through production of O_2^- and $OH^\cdot$ radicals). They therefore suggest that

production of cysteinyldopas *in vivo* may not necessarily be dependent on tyrosinase activity, as these other oxidising systems are known to occur in the cells of the body and may bring about these reactions *in vivo*.

Non-enzymic production of 5-S-CD has been demonstrated in non-melanin-producing tissues of the rat, mouse and cow, and in hair of ty-neg mice and white mice, completely lacking in follicular melanocytes (Fehling *et al.*, 1981; Ito *et al.*, 1983). Urinary excretion of 5-S-CD was found to be no different between black and albino mice.

It seems likely that the presence of protein-bound 5-S-CD in certain tissues of mice and rats, including albino mice, is due to its synthesis from protein-bound dopa, perhaps by one of the non-specific oxidation systems examined by Ito and Fujita (1981b). Protein-bound dopa is found in high concentrations in these tissues relative to the concentrations of free dopa, and is presumably produced by the action of tyrosine hydroxylase on tyrosine. Turn-over of the 5-S-CD-containing proteins would account for excretion of 5-S-CD in the urine. Although little is known of the situation occurring in humans, it may well be that a similar situation exists and that the 5-S-CD detectable in the plasma of ty-neg albinos is being produced in a similar manner to that suggested for albino mice. The likely existence of some non-tyrosinase mechanism for 5-S-CD production in ty-neg albinos brings into question the site and mechanism of production of 5-S-CD in normally pigmented individuals. It is possible that some 5-S-CD production in these individuals, rather than being mediated by tyrosinase in the melanocytes, occurs

in the same way as in albino subjects.

The presence of 5-S-CD in the urine of ty-neg albino negro subjects from Cameroon has been noted, as previously mentioned, and levels in these subjects found to be greater than the levels in normally pigmented negro subjects (Aquaron et al., 1981). The explanation offered for these findings was the existence of a block in the melanin pathway at some point distal to tyrosinase, perhaps by melatonin or dopachrome conversion factor, creating a build-up of pathway intermediates. The results of the study of ty-neg albino subjects presented here are dissimilar, in that levels of 5-S-CD in their plasma are no different from those seen in normally pigmented controls. Although plasma 5-S-CD has been shown to reflect melanocyte activity under certain conditions such as UVR exposure, it may be that normal baseline 5-S-CD levels do not exclusively reflect melanocyte activity, being perhaps due in part to non-tyrosinase oxidation of dopa at other sites in the body, and that it is only under abnormal or stimulated conditions that measurement of plasma 5-S-CD acts as an index of melanogenesis.

Plasma 5-S-CD in malignant melanoma

Malignant melanoma (MM), although the most malignant of skin tumours, if diagnosed and treated early, is curable. Obviously, therefore, the most important factor in the effective treatment of this tumour, in common with many other tumours, is its early recognition and removal (Pondes et al., 1981).

Study of patients with stage I primary melanoma demonstrated that plasma 5-S-CD levels were not significantly different from levels in normal healthy subjects or in subjects with a range of other primary tumours. Measurement of plasma 5-S-CD in these patients is, therefore, of no value in diagnosis of the primary tumour, and definitive identification depends upon surgical excision and histological examination of the excised tissue.

Agrup et al. (1977a) demonstrated normal urinary excretion of 5-S-CD in patients with primary melanoma. They also demonstrated that a proportion of patients with metastatic melanoma had raised urinary 5-S-CD concentrations, suggesting that measurement of urinary 5-S-CD may be of value in the diagnosis of secondary melanoma.

The value of plasma 5-S-CD measurements in patients being followed up after removal of primary melanoma or locally involved nodes was examined in a study of all patients attending the Dermatology melanoma follow-up clinic over a period of 1 year, all of whom were thought to be disease-free following surgery. Of the 88 patients in this group, 9 developed metastases either during the study period or in the 8 months following completion of the study. In 4 of these 9 patients raised plasma 5-S-CD levels were detected prior to the development of any other clinical or biochemical signs of metastatic

disease. The 2 patients whose 5-S-CD levels are presented in Fig.3.8 are included in this group. In the first of these patients (TI) this raised plasma 5-S-CD level was first observed when the patient was still apparently well and showing no signs of recurrence of the tumour. Two months after this first raised 5-S-CD level the patient began to develop clinical and other biochemical signs indicating the presence of secondary tumour, in this case the development of a right-sided pleural effusion and abnormal liver function tests, indicative of pulmonary and hepatic metastases. The patient died within a month of clinical diagnosis of metastatic disease.

The second patient (HI) whose plasma 5-S-CD levels are presented in Fig.3.8 was found to have a slightly raised 5-S-CD level on attendance at the Dermatology follow-up clinic in March '83. On 3 subsequent visits to the clinic her plasma 5-S-CD was found to be within the reference range. A second raised level, this time very markedly raised at 1.1μmol/l, was seen 8 months after the first raised level, in November. Shortly after detection of this very high 5-S-CD the patient began to feel generally unwell, with nausea, vomiting and abdominal pain. Further investigation showed pulmonary metastases on chest X-ray, and hepatic metastases were demonstrated by liver ultrasound and abnormal liver function tests. This patient died within a few weeks of clinical diagnosis of metastatic disease.

Both of these patients died approximately 2 months after the finding of a raised plasma 5-S-CD level. In the case of HI this was actually 10 months after the detection of a slightly raised 5-S-CD level which was not pursued, due to the finding of normal 5-S-CD levels on 3 subsequent occasions. Whether this particular

patient should have been further investigated on the basis of one slightly raised 5-S-CD level is difficult to say as it is not clear why a patient should have a raised level on one occasion and a normal level on the following occasion. If plasma 5-S-CD is related to the presence of clinically undetectable metastases, as seems likely, it may be that it reflects some growth spurt of the secondary tumour with production of melanin precursors and melanin.

The 2 remaining patients who were found to have raised 5-S-CD levels prior to the development of other signs of secondary melanoma, had these raised levels 2 and 9 months prior to clinical diagnosis of metastases, respectively. The raised 5-S-CD levels in both of these patients were only marginally elevated above the 20nmol/l upper limit, at 25·7 and 24·6 nmol/l, respectively. These raised levels were measured towards the end of the year in which these patients were studied, and so the progression of disease in these patients could not be followed. It may well have been the case that as the metastatic disease progressed and other signs became apparent, the plasma 5-S-CD levels became more markedly elevated. The further 3 patients who developed metastases in the 8 months following the study, who did not at any time during the study have elevated 5-S-CD levels, may also have had raised levels prior to the clinical detection of metastases which occurred at a time when plasma 5-S-CD levels were no longer being measured routinely and were, therefore, not detected.

There were a further 2 patients who developed metastases within the year of study who did not have raised plasma 5-S-CD levels even after metastases were clearly present. One of these patients had

a large abdominal secondary tumour removed which was barely pigmented and was found, on histological examination to contain little or no melanin pigmentation. A normal plasma 5-S-CD in this patient could, therefore, be simply explained in terms of lack of melanin production by the very undifferentiated tumour cells. Tumour tissue from the second patient who came into this category was not available for examination, so its pigmentary status remains unknown, although it would seem reasonable to predict that this tumour would also have been found to be amelanotic or weakly pigmented. Obviously it is not possible to formulate a hypothesis about the relationship between the pigmentary status of secondary melanoma and plasma 5-S-CD levels on the basis of the findings in one individual patient. However, Agrup et al. (1979a) found that normal urinary excretion of 5-S-CD in the presence of metastatic melanoma (which occurred in 10% of the patients whom they studied) did not have a single explanation. Some metastases in these patients were amelanotic or weakly pigmented, some were solitary and/or small, but normal values were also found in some patients with numerous pigmented metastases.

It is impossible to predict whether a patient will develop metastases producing massive amounts of melanin, or completely non-pigmented metastases on the basis of the nature of the primary tumour, as there appears to be no relationship between the two. Patients may have a deeply pigmented primary tumour which metastasises to produce an amelanotic secondary tumour. The only way to determine the pigmentary status of a secondary tumour is, therefore, to remove the tissue and examine it, both grossly and microscopically, for the presence of melanin. This procedure appeared to be performed rarely

in stage III melanoma patients as the nature of metastases is usually obvious clinically. It was, therefore, very difficult to obtain information on the pigmentation of the secondary tumour tissue.

Study of 26 patients with known stage III metastatic melanoma at the time of 5-S-CD measurement produced results in keeping with those from melanoma follow-up patients developing stage III melanoma during the course of study. Approximately 60 percent of the 26 patients studied were found to have raised plasma 5-S-CD levels, and in those from whom more than one sample was obtained for 5-S-CD estimation, all subsequent samples also had elevated 5-S-CD levels The remaining 40 percent of patients had normal 5-S-CD levels on each occasion in spite of the presence of metastatic tumour. A number of the patients in this group were known to have metastatic deposits in the liver, and examination of liver function in the 26 patients showed that of the 15 with raised plasma 5-S-CD levels, 11 also had abnormal liver funtion tests (LFTs), with either lactate dehydrogenase (LDH) or alkaline phosphatase (ALP), or both, being elevated. This compared with 2 out of 6 patients with normal 5-S-CD levels having abnormal LFTs. Results of LFTs were not available for the remaining 5 patients with normal 5-S-CD levels. These results could be interpreted as indicating some association between liver metastases and raised plasma 5-S-CD levels although there were, of course, 4 patients in whom an elevated 5-S-CD was not associated with abnormal LFTs. A possible relationship between liver metastases and plasma 5-S-CD levels suggests that the raised 5-S-CD levels may be due not simply to the presence of melanoma cells which are producing abnormally high levels of 5-S-CD, but that impairment of liver function, by the presence of metastatic deposits, may also be a contributory factor.

This in turn suggests a role for the liver in the normal metabolism of 5-S-CD, which is discussed in the following section. The possibility also exists that the finding of high 5-S-CD levels in these patients with abnormal liver function simply reflects high tumour bulk in these patients and an increased likelihood of spread to the liver.

These studies in melanoma patients demonstrated that whereas plasma 5-S-CD levels were not abnormal in patients with stage I primary melanoma, approximately 60 percent of patients with secondary melanoma had raised 5-S-CD levels. In a number of cases, 5-S-CD levels were shown to be elevated prior to clinical or other biochemical signs of metastatic disease. In 4 patients (1 follow-up patient and 3 with known stage III disease at time of study) it was possible to relate normal 5-S-CD levels to the amelanotic nature of the secondary tumour tissue, although in the majority of cases tumour tissue was not available for examination of its pigmentary status.

The results of the study of patients being followed up after surgery for stage I or stage II melanoma indicate that, in some cases, measurement of plasma 5-S-CD on attendance at the follow-up clinic can be a useful addition to clinical examination of the patient, and can occasionally indicate the presence of stage III melanoma prior to any clinical signs of progressing disease. The small number of patients in this group who developed stage III melanoma during the study, and the even smaller number in whom an increased 5-S-CD level was the first sign of spread of disease, suggest that the measurement of plasma 5-S-CD levels in all follow-up patients on each attendance at the clinic is probably not a worthwhile procedure. However, it

may be that plasma 5-S-CD estimation is a useful follow-up procedure in patients who had either a thick stage I primary tumour, or stage II melanoma, prior to surgery, as indicated by the proportion of patients who developed stage III melanoma and who had previously either a relatively thick stage I tumour (5 of the 7 in whom Breslow measurements were available had tumours thicker than 2mm), or stage II melanoma (1 of the remaining 2 patients had stage II melanoma prior to surgery).

Nevertheless liver function in a high proportion of patients with secondary melanoma and raised 5-S-CD levels, was impaired, suggesting a possible relationship between liver function and plasma 5-S-CD levels, and prompting further investigation of liver function and its relationship to 5-S-CD.

Plasma 5-S-CD and liver function

A group of patients with non-melanoma malignant tumours but without known liver involvement were studied. These tumours included breast, lung, ovary and stomach cancers, and lymphoma, and in all 38 patients 5-S-CD levels were found to be normal.

A further 10 patients with miscellaneous malignant disease were studied, all of whom had known metastatic involvement of the liver and deranged liver function. Although LFTs in these patients were abnormal, 5-S-CD levels in all patients were normal. This study does not support the theory that raised plasma 5-S-CD levels may be simply related to the presence of metastatic tumour in the livers of melanoma patients, despite the fact that in many cases their liver function was grossly abnormal.

The liver is a common site for metastatic spread of melanoma,

and it is possible that in patients with widespread secondary disease and multiple metastases, who have raised plasma 5-S-CD levels as a consequence of the high tumour load present, the finding of metastatic tumour in the liver is highly probable, resulting in abnormal liver function which is not directly related to the level of 5-S-CD in the plasma.

Abnormal ALP levels in 2 of the 6 patients with stage III melanoma and normal plasma 5-S-CD levels may indicate the presence of widespread amelanotic or weakly pigmented tumour in these patients.

To examine further the handling of 5-S-CD by the liver and the effect of deranged liver function on plasma 5-S-CD levels, a group of patients with liver disease, in the form of chronic hepatitis or cirrhosis, was examined. All of these patients had markedly abnormal LFTs. Measurement of plasma 5-S-CD levels in these patients demonstrated that all but 3 of the 14 patients in this study had normal 5-S-CD levels, and 2 of the 3 elevated levels were only very slightly raised above the limit of 20 nmol/l. The third abnormal 5-S-CD was greater than 3 times this figure at 62·5 nmol/l. The patient in whom this level was seen had alcoholic cirrhosis and highly abnormal LFTs at the time of this 5-S-CD measurement. He was seen at monthly intervals over the following 2 months and had 5-S-CD and LFT measurements performed on each occasion. His bilirubin and ALP levels remained considerably elevated over this period, but his 5-S-CD was seen to return to normal. The patient had no history of malignant melanoma, was apparently on no medication which would account for this raised 5-S-CD, and had not recently been exposed to large amounts of UVR. The explanation for the raised

5-S-CD in this man is not clear. However, the finding of elevated plasma 5-S-CD levels in patients with abnormal liver function appeared to be very uncommon, and it seemed that in general, abnormal liver function does not cause elevated plasma 5-S-CD levels, and that the apparent relationship between these 2 factors in stage III melanoma is simply due to high tumour load in these patients.

The role of the liver in 5-S-CD metabolism has been investigated (Agrup et al., 1977b). Catechol-O-methylation is known to be important in the metabolism of dopa, and large amounts of the product of this reaction, 3-methoxytyrosine, have been detected in the urine of patients with malignant melanoma. Large amounts of dopa are also decarboxylated to form dopamine, which is also detectable in the urine. These two reactions are shown in Fig.4.1. Incubation of 5-S-CD with rat liver extract containing large amounts of catechol-O-methyl transferase and S-adenosylmethionine, has been shown to result in the production of a methyl derivative of 5-S-CD, identified by gas chromatography-mass spectrometry (GC-MS). This same methyl derivative was also detected in large amounts in the urine of 2 melanoma patients. Incubation of 5-S-CD with guinea-pig kidney extract containing dopa decarboxylase did not result in the formation of 5-S-cysteinyldopamine detectable by GC-MS, nor could any of this compound be demonstrated in the urine of the 2 melanoma patients.

It appears from these studies that metabolism of 5-S-CD is largely by methylation, with decarboxylation playing a minor role, if involved at all. Although large amounts of the methyl derivative of 5-S-CD were found in the urine of melanoma patients, it is not clear in what proportion this compound is present relative to

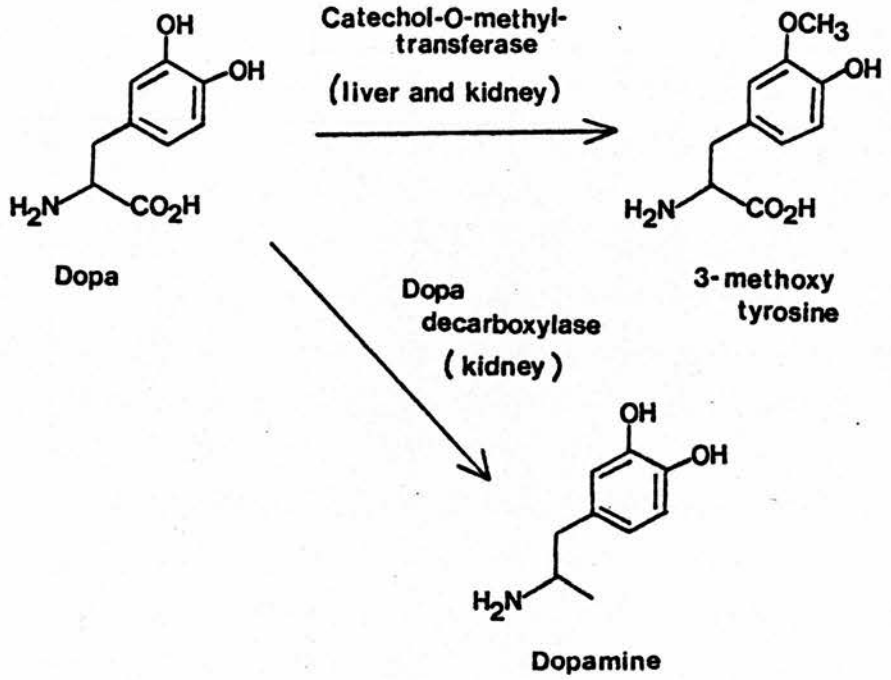

FIGURE 4.1 Metabolism of dopa in the liver and kidney

the unmetabolised 5-S-CD. Whether O-methylation plays a major role in the handling of 5-S-CD by the body under normal conditions is not clear, and whether the relative amounts of 5-S-CD and its methyl derivative excreted under conditions of abnormal liver function are different to those found in normal liver function is unknown.

Plasma 5-S-CD and renal function

5-S-CD is excreted in relatively large amounts in urine, as compared to the low levels present in the plasma of healthy individuals, which are undetectable by fluorimetric measurement (Agrup et al., 1975b).

Calculation of renal clearance of 5-S-CD in melanoma patients with high plasma 5-S-CD levels has suggested that approximately 50 percent of the 5-S-CD filtered in the glomeruli is excreted in the urine, unlike most amino acids which are more effectively reabsorbed by the kidney tubules (Agrup et al., 1975a). Whether a similar situation occurs in healthy individuals with much lower plasma levels of 5-S-CD is unknown. The kidney, however, obviously plays a major role in the handling of 5-S-CD by the body, excreting both 5-S-CD and its O-methyl derivative in relatively large amounts.

Renal function, and its effect on plasma 5-S-CD levels was investigated in 14 patients with miscellaneous forms of chronic renal failure. Results from these patients demonstrated a strong correlation between glomerular function, as estimated by plasma creatinine levels, and plasma 5-S-CD concentrations. The plasma 5-S-CD levels seen in some of these patients were more than twice the upper limit of 20nmol/l, and only 5 of the patients had levels which fell within the reference range. Clearly, impairment of glomerular function is able to have a significant effect on plasma 5-S-CD levels. This may be directly due to decreased glomerular filtration in these patients, as suggested by the relationship between plasma 5-S-CD and creatinine levels. However, it may indicate that the kidney has some role to play in metabolism of 5-S-CD, since large amounts of

catechol-O-methyl transferase are known to be present in the kidney.

Hansson et al. (1979b) have studied serum levels of dopa and a number of dopa metabolites in uraemia and in patients on renal dialysis. Serum concentrations of 5-S-CD, but not dopa, were found to be increased in renal insufficiency, possibly explained by the fact that dopa, unlike 5-S-CD, is decarboxylated. Hansson et al. also found that plasma clearance of 5-S-CD was more impaired than creatinine clearance in patients with chronic renal failure. They concluded that the changes in serum concentrations of 5-S-CD and related compounds in renal failure were probably due directly to the impaired renal function, but that indirect mechanisms may also be of importance.

Impaired renal function was considered as a possible contributory factor in earlier studies of melanoma patients with highly elevated plasma 5-S-CD levels. Renal function, however, unlike liver function, was not commonly impaired in our patients. However, the fact that impairment of renal function can cause such marked abnormalities in plasma 5-S-CD levels suggests that this is a factor which should be considered in melanoma patients with elevated 5-S-CD levels, as it may produce misleading results in patients suspected of having metastatic disease.

Plasma 5-S-CD in Parkinson's disease

The finding of a highly elevated plasma 5-S-CD level (172 nmol/l) in a melanoma follow-up patient led to the assumption that this patient had developed metastatic melanoma. However, this patient also had Parkinson's disease, which was being treated with the drug Sinemet, a combination of L-dopa and carbidopa.

Parkinson's disease, a neurological condition involving muscular rigidity, hypokinesis and tremor, has long been known to be related to abnormal function in the basal ganglia. The basal ganglia receive a strong dopaminergic innervation, and the disease is associated with a reduced amount of dopamine in the brain. Dopamine itself does not penetrate the blood-brain barrier, so as such cannot be used directly to supplement the reduced levels in these patients. L-dopa, however, can penetrate the blood-brain barrier and can be used to good effect. Peripheral conversion of administered L-dopa to dopamine by dopa decarboxylase may, however, lead to a number of unwanted side effects. This problem can be overcome by administration of a dopa decarboxylase inhibitor, such as carbidopa, in conjunction with the L-dopa, for instance in the form of the drug Sinemet.

The finding of a raised plasma 5-S-CD in one patient being treated with this drug prompted the study of other patients undergoing the same treatment. A further 5 patients undergoing treatment with Sinemet were found to have elevated 5-S-CD levels, compared with 3 patients with Parkinsonism being treated with amantadine, all of whom had normal 5-S-CD levels. It was not possible to obtain samples from patients with untreated Parkinsonism. The findings of raised plasma 5-S-CD levels in these patients is in keeping with the finding of elevated urinary excretion of 5-S-CD in patients undergoing treatment with dopa and carbidopa (Stewart et al., 1983). The obvious explanation for the raised plasma 5-S-CD levels in patients being treated with Sinemet is one of increased availability of substrate for 5-S-CD production. The large amount of L-dopa

being given to the patient is not decarboxylated to dopamine, a major metabolite, due to the presence of carbidopa, and is available for incorporation into other biochemical pathways, for instance the tyrosinase pathway, leading to the production of 5-S-CD (Fig.4.2).

Whether this increased production of 5-S-CD is due to formation by the melanocytes via the tyrosinase pathway, which would require uptake of the dopa by these cells, or whether this represents some non-tyrosinase production of 5-S-CD, as discussed on page 136, is not clear. Whatever the mechanism, this finding of high 5-S-CD levels in patients being treated with L-dopa points to a further potential source of false positively high 5-S-CD levels in patients with melanoma, and, although likely to be a very rare finding, the presence of one such patient in the study of melanoma follow-up patients suggests that this possibility should not be forgotten in any studies of such patients.

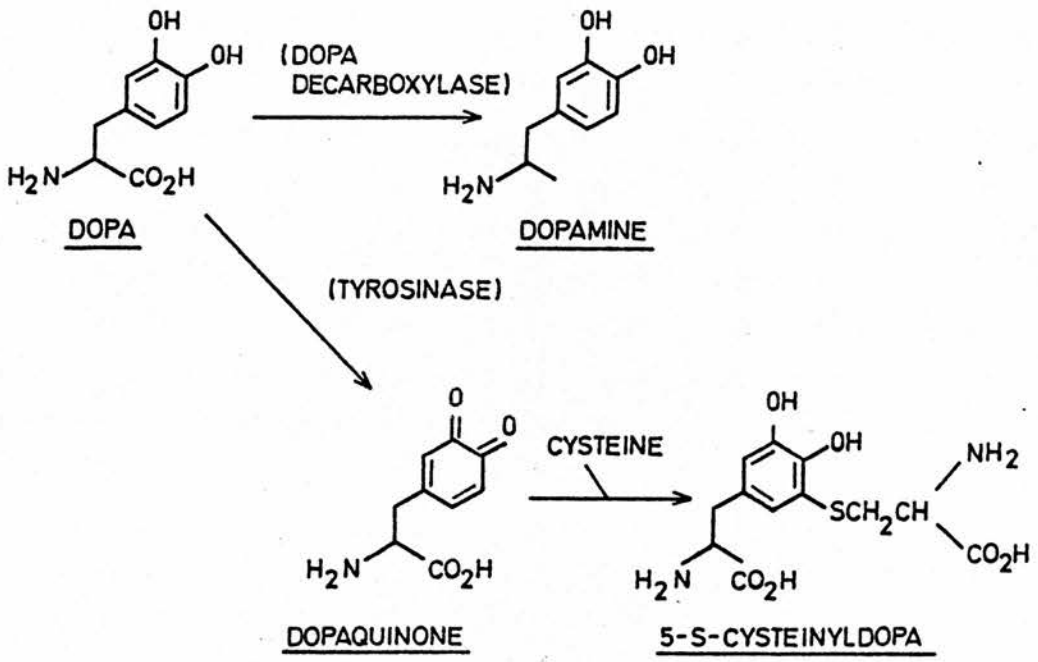

FIGURE 4.2 Metabolism of dopa by tyrosinase and dopa decarboxylase

Conclusions

1) Plasma 5-S-CD levels in healthy individuals are not greatly affected by unavoidable sun exposure in a temperate climate such as that of S.E. Scotland, and studies of 5-S-CD levels in this climate need not, therefore, be restricted to those months of the year when sunlight is minimal.

2) Plasma 5-S-CD levels can be greatly influenced by exposure to UVR therapy (UVB) or photochemotherapy (PUVA). Patients with psoriasis treated with these types of UVR developed a near two-fold increase in their plasma 5-S-CD level within the first 5 treatments, before pigmentation developed, with subsequent increments of up to 4 times the pre-treatment level being found in the PUVA group.

3) Dithranol treatment caused an increase in plasma 5-S-CD in some psoriatic patients.

4) Differences in skin and hair colour do not appear to result in differences in plasma 5-S-CD levels.

5) Measurement of plasma 5-S-CD is of no value in the diagnosis of stage I melanoma.

6) Plasma 5-S-CD is raised in 60 percent of patients with stage III melanoma. Raised values are independent of the site of the metastases. Normal 5-S-CD levels in patients with stage III melanoma may be related to the amelanotic nature of the secondary tumour tissue in these patients.

7) 10 percent of treated stage I and stage II melanoma patients developed stage III melanoma during a study period of 20 months, whilst undergoing follow-up. In approximately half of these patients a raised plasma 5-S-CD level was observed prior to clinical detection of metastases.

8) Plasma 5-S-CD levels are not raised in patients with non-melanoma tumours of the types studied.

9) Plasma 5-S-CD is rarely raised in patients with chronic inflammatory liver disease.

10) Plasma 5-S-CD may be raised in patients with poor glomerular function.

11) Plasma 5-S-CD levels may be greatly increased by the administration of L-dopa, as seen in a number of patients being treated for Parkinson's disease.

12) Plasma 5-S-CD levels in tyrosinase-negative albino patients are no different from those in normally pigmented controls, suggesting production of 5-S-CD by a non tyrosinase-dependent process.

APPENDIX I

CHEMICALS

The following list contains all chemicals and solvents used in these studies, and their suppliers.

Bio-Rad Laboratories Ltd., Watford, Herts., U.K.

AG50W-X8 cation exchange resin (200-400, H^+).

Camlab Ltd., Cambridge, U.K.

Woelm Neutral Activity Grade I aluminium oxide.

Fluorochem Ltd., Glossop, Derbyshire, U.K.

Methanesulphonic acid (puriss).

MacFarlane Robson Ltd., Glasgow, U.K. (BDH suppliers)

Acetic acid; 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris); disodium hydrogen orthophosphate; ethylenediamine tetraacetic acid (EDTA); hydrochloric acid; orthophosphoric acid; perchloric acid; potassium dihydrogen orthophosphate; potassium iodide; sodium acetate trihydrate; sodium hydroxide; sodium sulphate. All BDH chemicals were AnAlar grade.

May and Baker Ltd., Dagenham, Essex, U.K.

Iodine (resublimed).

Scotlab Instrument Sales Ltd., Carluke, Lanarkshire, U.K.

Fisons HPLC grade methanol.

Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

D-cysteine; L-cysteine; DL-dithiothreitol; D-β-3,4-dihydroxyphenylalanine (dopa); glutathione (reduced); mushroom tyrosinase.

Shandon Southern Products Ltd., Runcorn, Cheshire, U.K.

ODS-hypersil reverse-phase column packing (5μm).

APPENDIX II

BUFFERS

The following are the buffers used in these studies.

Sorensens phosphate buffer, pH 6·5

0·5M Na_2HPO_4 titrated with 0·5M KH_2PO_4 to give a pH of 6·5.

Tris buffer, pH 8·6

1M Tris (2-amino-2-(hydroxymethyl)-propane-1,3-diol) titrated with conc. HCl to give a pH of 8·6.

The above buffers were prepared at 20°C.

APPENDIX III

This appendix lists the abbreviations used in this thesis. The abbreviations are defined both here and the first time they appear in the text.

ACTH	adrenocorticotrophic hormone
ALM	acral lentiginous melanoma
ALP	alkaline phosphatase
ALT	alanine amino transferase
CAT	computerised axial tomography
CV	coefficient of variation
dopa	dihydroxyphenylalanine
DPPH	diphenylpicrylhydrazyl
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
HPLC	high-performance liquid chromatography
LFT	liver function test
LMM	lentigo maligna melanoma
MM	malignant melanoma
8-MOP	8-methoxypsoralen
MSH	melanocyte stimulating hormone
NM	nodular melanoma
OCA	oculocutaneous albinism
PUVA	psoralen and UVA
5-S-CD	5-S-cysteinyldopa
5-S-D-CD	5-S-D-cysteinyl-L-dopa

SSM	superficial spreading melanoma
ty-neg OCA	tyrosinase-negative oculocutaneous albinism
ty-pos OCA	tyrosinase-positive oculocutaneous albinism
UVA	long-wave ultraviolet radiation
UVB	short-wave ultraviolet radiation
UVR	ultraviolet radiation

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PUBLICATIONS

The following paper was published as a result of work presented in this thesis.

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Plasma 5-S-Cysteinyldopa Concentrations in Oculocutaneous Albinism

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Nimmo JE, Hunter JAA, Percy-Robb IW, Jay B, Phillips CI, Taylor WOG. Plasma 5-S-cysteinyldopa concentrations in oculocutaneous albinism. Acta Derm Venereol (Stockh) 1985; 65: 169–171.

5-S-cysteinyldopa concentrations were determined by high-pressure liquid chromatography and electrochemical detection in plasma from normally pigmented patients and patients with oculocutaneous albinism, both tyrosinase-positive and tyrosinase-negative. The plasma 5-S-cysteinyldopa concentrations were similar in all three groups, suggesting that 5-S-cysteinyldopa can be produced by mechanisms which do not involve tyrosinase.
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Human oculocutaneous albinism is of two main types: tyrosinase-positive and tyrosinase-negative albinism. These can be distinguished on the basis of both clinical features, and presence or absence of tyrosinase activity in the hairbulbs (1).

Tyrosinase is thought to be essential for the first two steps in melanin synthesis. These are the hydroxylation of tyrosine to form dihydroxyphenylalanine (dopa) and the subsequent oxidation of dopa to form the highly reactive intermediate dopaquinone. The pathway leading to formation of the red/yellow phaeomelanins involves the spontaneous coupling of dopaquinone with cysteine to form a number of different cysteinyldopas of which quantitatively the major component is 5-S-cysteinyldopa (5-S-CD) (2).

5-S-CD can be detected in the urine of normal healthy individuals, regardless of skin or hair colour (3). Patients lacking demonstrable tyrosinase activity would be expected to be incapable of synthesising melanin or its precursors, including 5-S-CD.

We report the results of a study in which we measured plasma 5-S-CD concentrations in both tyrosinase-positive and tyrosinase-negative patients with oculocutaneous albinism to see if it was possible to distinguish between the two groups on the basis of this measurement.

MATERIALS AND METHODS

There were twenty patients in the study, all with oculocutaneous albinism. They were divided into ten tyrosinase-positive and ten tyrosinase-negative albinos, on the basis of clinical assessment either on its own or, in a few cases, accompanied by a hairbulb tyrosinase test (4).

Controls were ten healthy volunteers of both sexes with a wide range of hair colour.

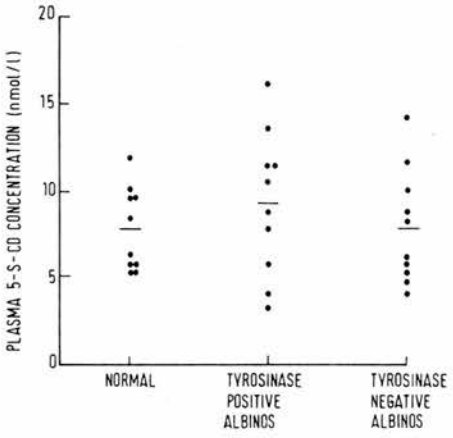

Fig. 1. Plasma 5-S-CD concentrations in albinos and controls.

Sampling from these 3 groups was not restricted to any particular time of year, as we had previously demonstrated that the increases in plasma 5-S-CD levels observed during the summer months in Edinburgh, although significant, were not of sufficient magnitude to invalidate any results obtained during these months (5).

Blood samples were collected into lithium heparin tubes containing dithiothreitol to a final concentration of 5 mmoles/l. 5-S-CD was extracted from the plasma by adsorption onto alumina at pH 8.6 followed by elution with 0.2 M $HClO_4$. 5-S-CD determination was by high-pressure liquid chromatography (HPLC) with electrochemical detection (6), and using 5-S-D-cysteinyl-L-dopa as internal standard.

RESULTS

The plasma concentrations of 5-S-CD found in controls and albino patients are given in Fig. 1.

Statistical analysis of the results using an independent *t*-test, showed that the three groups of results were not significantly different from one another.

DISCUSSION

We have found no significant difference between plasma concentrations of 5-S-CD in normally pigmented controls and in patients with oculocutaneous albinism, or between 5-S-CD concentrations in tyrosinase-positive and tyrosinase-negative albinos.

The finding of measurable, and indeed apparently normal concentrations of 5-S-CD in plasma from tyrosinase-negative albinos is perhaps surprising in the light of a demonstrable lack of active tyrosinase in the hairbulbs of these patients (1). However, Aquaron et al. were able to demonstrate urinary excretion of 5-S-CD in both tyrosinase-positive and tyrosinase-negative albino negroes in the sunny climate of Cameroon (7).

5-S-CD has been shown to be present in non-melanogenic tissues, such as liver, kidney and brain, of mice and rats, as well as in hair of albino mice (8). In these cases the amino acid is present mainly in a protein-bound form, free 5-S-CD being excreted in the urine. The finding of 5-S-CD in these tissues, in which tyrosinase is not detectable, indicates that it can be produced by some oxidation mechanism which clearly does not involve tyrosinase, although the importance of this enzyme in melanin production within melanocytes is not open to question (2).

Similar tyrosinase-independent pathways have also been proposed as a result of work on guinea-pig tissues, including kidney, spleen, heart and sympathetic ganglia, and on the

ganglion stellatum of the cow (9, 10). Ito et al. have, in fact, demonstrated the formation of cysteinyldopas in vitro from dopa and cysteine by a number of systems not involving tyrosinase, including peroxidase-H_2O_2, and superoxide and hydroxyl radicals (11, 12, 13).

It seems likely that the presence of protein-bound 5-S-CD in tissues of mice and rats, including the albino mouse, is due to its synthesis from protein-bound dopa, perhaps by one of the non-specific oxidation mechanisms previously mentioned. Protein-bound dopa is found in high concentrations in these tissues relative to the concentrations of free dopa, and is presumably produced by the action of the enzyme tyrosine hydroxylase on tyrosine. Turnover of the 5-S-CD-containing proteins would account for the excretion of the amino acid in the urine of the rats and mice studied, including the tyrosinase-negative albino mice, in which urinary excretion of 5-S-CD was found to be very similar to that in pigmented animals. Without further investigation in humans it is difficult to explain our observations in tyrosinase-negative albinos, but production of 5-S-CD in these individuals would certainly appear to be by some non-tyrosinase-dependent oxidation pathway, and could perhaps involve one or more of those suggested by 5-S-CD production in non-melanogenic tissues. Reactive oxygens, for instance, are known to be produced in many biological processes.

The findings in human albinos are certainly comparable to findings in albino mice, and it may be that similar mechanisms for production of 5-S-CD are operating in the two species.

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